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(54) Title: RECOMBINANT CYTOMEGALOVIRUS EXPRESSION SYSTEM TARGETING THE IMMEDIATE-EARLY GENE REGION

pGEM11Zf-K181H3L

pMV11 + gene cassette

↓

Hpa I site modified to contain
Not I restriction site or att I site
To facilitate additional cloning.

Promoter = HCMV IE1 or other promoter
Gene = ZP3 or OVATfR or HA cDNA with cell membrane anchoring domain or IL-6 cDNA
P[A] = SV40 polyadenylation sequence

(57) Abstract

The present invention consists in a recombinant cytomegalovirus comprising: a cytomegalovirus genome including within the immediate-early (IE) gene region of the virus genome, a genetic cassette including at least a promoter adjacent to at least a DNA sequence and a polyadenylation stop sequence, wherein (1) the promoter is adapted to drive the expression of the DNA sequence, and (2) the promoter, DNA sequence and the polyadenylation stop sequence is heterologous to the virus in which the cassette is inserted.

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**RECOMBINANT CYTOMEGALOVIRUS EXPRESSION SYSTEM TARGETING
THE IMMEDIATE-EARLY GENE REGION**

Field of the invention

The present invention relates to a recombinant cytomegalovirus (CMV) vector and in particular to a recombinant CMV which includes at least a heterologous DNA
5 sequence within the genomic region encoding for immediate-early proteins.

General

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variation and
10 modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in the specification, individually or collectively, and any and all combinations or any two or more of the steps or features.

The present invention is not to be limited in scope by the specific embodiments
15 described herein, which are intended for the purpose of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention as described herein.

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description. All references cited, including patents
20 or patent applications are hereby incorporated by reference. No admission is made that any of the references constitute prior art.

As used herein the term "derived from" shall be taken to indicate that a specific integer may be obtained from a particular source *albeit* not necessarily directly from that source.

25 Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Background to the Invention

Cytomegaloviruses (CMV) are a subfamily of Herpesviridae (commonly known as the herpes viruses). They exhibit a narrow host range, there is a long duration in their replication cycle frequently resulting in slowly progressing lytic foci in cell culture, and they often produce enlarged cells (cytomegalia) both *in vitro* and *in vivo*.

CMVs have a genomic organisation that is distinct from other herpes virus groups. The 230-kb dsDNA genome of human CMV (HCMV) was sequenced in about 1990 and has at least 200 open reading frames (ORFs). The function of some HCMV proteins are known or predicted due to their homology with other viral and cellular proteins. However, for the majority of the HCMV ORFs, the function(s) of the proteins they encode is unknown. Murine CMV (MCMV) has also been sequenced and is closely related to HCMV. Seventy-eight of the 170 MCMV ORF exhibits sequence similarity with HCMV.

Viral delivery systems have been used for many years, beginning with vaccinia virus recombinant studies. In those studies vaccinia virus was genetically manipulated to express heterologous antigens. While the concept of using viruses as a delivery system for heterologous genes and or antigens became obvious with those studies, what was not obvious was the answer to a more practical question of which are the best candidate virus vectors. In answering this question, details of the pathogenicity of the virus, its site of replication, the kind of immune response it elicited, the potential for it to express foreign antigens and its suitability for genetic engineering, are all factors in the selection. For example, a viral vector carrying a therapeutic agent needs to target the correct cell type to deliver the therapeutic agent. One viral subfamily that has received little attention as a possible vector system are cytomegaloviruses.

The present invention seeks to provide a CMV vector that ameliorates some or all of the problems associated with the prior art.

Summary of the Invention

The present invention consists in a recombinant cytomegalovirus comprising: a cytomegalovirus genome including within the immediate-early (*IE*) gene region of the virus genome, a genetic cassette including at least a promoter adjacent to at least a DNA sequence and a polyadenylation stop sequence, wherein (1) the
5 promoter is adapted to drive the expression of the DNA sequence, and (2) the promoter, DNA sequence and the polyadenylation stop sequence is heterologous to the virus in which the cassette is inserted.

In a preferred embodiment, the invention consists of a recombinant
10 cytomegalovirus comprising: a cytomegalovirus genome including within the immediate-early (*IE*) gene region of the virus genome, a genetic cassette including at least a HCMV *IE*-1 promoter adjacent to at least a DNA sequence and a polyadenylation stop sequence, wherein (1) the HCMV *IE*-1 promoter is adapted to drive the expression of the DNA sequence, and (2) both the DNA sequence
15 and the polyadenylation stop sequence are heterologous to the virus in which the cassette is inserted.

In another embodiment of the invention the cassette is inserted into the *IE* gene region in such a manner that it inactivates at least a gene that encodes for an *IE* protein. Desirably, insertion of the cassette into the viral genome leads either to
20 activation of an identifiable phenotype or its deletion from the viral genome therein providing a means for selecting for the transformed virus.

The heterologous DNA sequence(s) employed in the cassette may encode part or all of any natural or recombinant protein that is heterologous to the CMV species from which the recombinant virus is formed. Preferably, the heterologous DNA
25 sequence encodes one or more of the following: zona pellucida (ZP) protein(s); malarial surface antigen; beta-galactosidase; major antigenic viral antigen(s) (eg Haemagglutinin (HA) from influenza virus); eukaryotic polypeptide(s); enzyme inhibitor(s); hormone(s); lymphokine(s); cytokine(s); chemokine(s); plasminogen activator(s); or natural, modified or chimeric immunoglobulin or a fragment
30 thereof.

In a highly preferred form of the invention the heterologous DNA sequence encodes either one or more of the ZP proteins or a viral antigen like HA.

The present invention also provides a prophylactic or therapeutic substance that comprises a recombinant virus vector produced according to the present invention
5 in a pharmaceutically acceptable vehicle. In a preferred form of the invention the pharmaceutically acceptable vehicle may also comprise one or more excipients, adjuvants, stabiliser or other like substances. A pharmaceutical of this type should be prepared and used according to standard techniques known in the art.

The present invention also provides a a method for manufacturing a prophylactic
10 or therapeutic substance, comprising the steps of:

- a) growing the recombinant CMV as herein described in a suitable host cell system; and
- b) mixing the resulting virus in an effective immunising amount with a pharmaceutically acceptable excipient.

15 Detailed Description of the Invention

The present invention employs the use of CMV as a vector for the delivery of polypeptides and therapeutic agents. CMV is well suited for this role because it is ubiquitous in nature; it is a DNA virus; non-mutagenic; non-integrative; stable; it has benign effects in healthy individuals; and species specific CMV are commonly
20 found in most animal populations. Immunoglobulin responses to the expressed antigens are predominantly of a IgG2a isotype, predictive of a Type 1 immune response. Furthermore, inoculation of animals with CMV generates a long lasting IgA response in the salivary gland.

The present inventors have found that a recombinant CMV produced in
25 accordance with the present invention can boost an existing immune response to the expressed antigen to give long lasting IgA responses in the salivary gland. Responses can be detected in the absence of significant detectable virus replication in immunologically competent animals.

Thus present invention consists in a recombinant cytomegalovirus comprising: a
30 cytomegalovirus genome including within the immediate-early (IE) gene region of

the virus genome, a genetic cassette containing at least a promoter adjacent to at least a DNA sequence and a polyadenylation stop sequence, wherein (1) the promoter is adapted to drive the expression of the DNA sequence, and (2) the promoter, DNA sequence and the polyadenylation stop sequence are
5 heterologous to the virus in which the cassette is inserted.

Without wishing to be limited to any mode of action the inventors believe that expression of foreign proteins via recombinant virus (rMCMV) produced according to the present invention generates an immune response to the protein, which results in increased recognition and clearance of host cells expressing the
10 membrane-bound protein and hence also clears the virus. In effect, foreign protein most likely acts as an additional virus antigen, which then overrides the immune evasion mechanisms developed by the virus. This "immunological attenuation" is generated through the expression of the heterologous antigen on the membrane of the infected host cell. This allows for increased recognition of
15 virus infected cells by the immune system.

The inventors have found that rMCMV-ovalbumin (OVA) and rMCMV-HA replicate in the salivary glands of IFN-alpha/gamma receptor knock-out mice, to levels comparable to those attained by parental (RM427+ or K181-WT) MCMV in immunocompetent animals. rMCMV-OVA also replicates in the salivary gland of
20 IFN-alpha R ko mice. These mice are defective in their immune responses. This suggests that rMCMV is able to replicate in salivary glands once the host immune response is defective. More specifically, it appears to be an early/innate immune response that is responsible for controlling virus replication.

rMCMV-OVA and rMCMV-HA both generate an immune response to OVA and HA
25 antigens, respectively, in the immunocompetent BALB/c mice. The predominant antibody response is of the IgG2a isotype, indicating a Th1-type response. This indicates that low levels of rMCMV replication are sufficient to generate an antibody response to the expressed foreign antigen.

Salivary gland rMCMV-OVA derived from the IFN-alpha/gamma R ko mice when
30 passaged through IFN-alpha R ko mice or BALB/c weanling mice, in an attempt to generate SGV show no virus. These experiments demonstrate that although

rMCMV is able to replicate in the salivary glands of immunodeficient mice it still cannot replicate in BALB/c salivary glands, which indicates that there is no deleterious mutation elsewhere on the MCMV genome responsible for reduced replication. These mutations could have been introduced and selected for during
5 the process of homologous recombination and subsequent isolation of the recombinants through tissue culture based techniques.

Preferably, the cassette is inserted into the *IE* gene region in such a manner that it inactivates at least a gene which encodes for an *IE* protein. In a highly preferred form of the invention the recombinant cytomegalovirus into which the cassette is
10 inserted contains an identifiable phenotype such as lac Z or other antibiotic resistance markers. Desirably, insertion of the cassette into the viral genome leads either to activation of the identifiable phenotype or its deletion from the viral genome therein providing a means for selecting for the transformed virus.

The cassette employed in the present invention may be inserted into the CMV
15 genome at any position that does not disrupt virus growth or replication. Preferably, the cassette is inserted into the CMV genome within or between the *IE* genes in the selected CMV. To the extent that the cassette is inserted within one or more of the *IE* genes in the viral genome the insertion should not disrupt virus growth or replication. For example, in mice, deletion of part or the entire *IE*-2 gene
20 does not appear to significantly disrupt MCMV growth or replication patterns. In humans, however, deletion of the same gene significantly alters HCMV replication properties. To the extent that the cassette is inserted between the *IE* genes in the viral genome, again the insertion should not disrupt virus growth or replication. In mice, this may be achieved by inserting the cassette either between genes *IE*-1
25 and *IE*-2 or between genes *IE*-2 and *IE*-3. In humans, this may be achieved by inserting the cassette between genes *IE*-1 and *IE*-2 or after the *IE*-2 gene.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation in
30 eukaryotic cells, with or without a CCAAT box sequence and additional regulatory elements (ie. upstream activating sequences, enhancers and silencers). Promoters may also be lacking a TATA box motif, however comprise one or more

"initiator elements" or, as in the case of yeast-derived promoter sequences, comprise one or more "upstream activator sequences" or "UAS" elements. For expression in prokaryotic cells such as bacteria, the promoter should at least contain the -35 box and -10 box sequences.

- 5 A promoter is usually, positioned upstream or 5', of the heterologous DNA sequence, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene.

In the present context, the term "promoter" is also used to describe a synthetic or
10 fusion molecule, or derivative that confers, activates or enhances expression of the heterologous DNA sequence in a cell. Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression of the gene and/or to alter the spatial expression and/or temporal expression.

- 15 Placing a heterologous DNA sequence operably under the control of a promoter sequence means positioning the said sequence such that its expression is controlled by the promoter sequence. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately
20 the same as the distance between that promoter and the gene it controls in its natural setting, ie., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous DNA sequence to be placed under its
25 control is defined by the positioning of the element in its natural setting, ie. the gene(s) from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in regulating the expression of the heterologous DNA sequence include viral, fungal, yeast, insect, animal and plant
30 derived promoters. Preferred promoters are capable of conferring expression in a eukaryotic cell, especially mammalian cell. The promoter may regulate the

expression of a gene constitutively, or differentially with respect to the tissue in which expression occurs or, with respect to the developmental stage at which expression occurs, or in response to external stimuli such as environmental stress, or hormones amongst others.

- 5 Those skilled in the art will recognise that the choice of promoter will depend upon the nature of the cell being transformed and the heterologous DNA sequence to be expressed. Such persons will be readily capable of determining functional combinations of minimum promoter sequences and operators for cell types in which the inventive method is performed.
- 10 In a particularly preferred embodiment, the promoter is a viral promoter, mammalian promoter, a bacterial or bacteriophage promoter sequence. In a highly preferred form of the invention the promoter is the *CMV* promoter sequence, more preferably the *CMV-IE* promoter or alternatively, the *SV40 promoter* and, in particular, the *SV40* late promoter sequence. These and other
- 15 promoter sequences suitable for expression of genes in mammalian cells are well known in the art.

- Thus, in one embodiment, the present invention consists in a recombinant cytomegalovirus comprising: a cytomegalovirus genome including within the immediate-early (*IE*) gene region of the virus genome, a genetic cassette
- 20 containing at least a HCMV *IE*-1 promoter adjacent to at least a DNA sequence and a polyadenylation stop sequence, wherein (1) the HCMV *IE*-1 promoter is adapted to drive the expression of the DNA sequence, and (2) both the DNA sequence and the polyadenylation stop sequence are heterologous to the virus in which the cassette is inserted.
- 25 While the cassette used in the present invention possesses its own promoter, it need not necessarily include other regulatory elements (eg enhancers) to promote gene expression. When the cassette does not contain such regulatory elements, the cassette is preferably inserted into the viral genome in such a manner ensuring that the endogenous viral regulatory elements influence expression of
- 30 the heterologous DNA sequence in the cassette via the HCMV *IE*-1 promoter. By way of example, high constitutive levels of expression may be achieved by

inserting the HCMV *IE-1* promoter adjacent to the MCMV *IE-1/ IE-2/ IE-3* enhancer. In a preferred form of the invention the cassette includes all of the regulatory elements to enhance expression via the HCMV *IE-1* promoter. Such elements will be well known to those of ordinary skill in this field. The techniques employed to insert such sequences into the viral vector and make other alterations in the viral DNA, e.g., to insert linker sequences and the like, are known to one of skill in the art [i]. Thus, given the disclosures contained herein the construction of suitable CMV vectors for expression of heterologous DNA sequences is within the skill of the art.

- 10 To maximise the stability and effective termination of the message encoded by the heterologous DNA sequence the cassette also includes a heterologous polyadenylation stop sequence. A person of ordinary skill in the field will appreciate that any polyadenylation stop sequence may be used in the present invention. Desirably, the polyadenylation stop sequence is derived from the SV40
15 polyadenylation stop sequence; human or bovine growth factor p[A], retroviral 3' LTR, together with any other long lived hormonal (cytokine for eg) polyadenylation stop sequence.

According to one form of the present invention the heterologous DNA sequence encodes at least a polypeptide. The term "polypeptide" as used herein includes
20 within its scope parts of polypeptides such as peptides, oligopeptides as well as complete or substantially complete proteins.

- It will be appreciated that the heterologous DNA sequence(s) employed in the cassette may encode part or all of any natural or recombinant protein that is heterologous to the CMV species from which the recombinant virus is formed.
25 For example, the heterologous DNA sequence might encode a polypeptide which consists of a plurality of antigenic and/or immunogenic peptides linked together in such a way that each of the peptides retains its immunological identity when expressed in a protein. Alternatively, the heterologous DNA sequence may encode one or more of the following, by way of example: ZP proteins; malarial surface antigens; beta-galactosidase; major viral antigen eg HA from influenza
30 virus or in the case of human immunodeficiency virus (HIV) the heterologous DNA sequence might encode for HIV gp 120 and or the DNA sequence for the HIV gag

protein; eukaryotic polypeptide such as, for example, a mammalian polypeptide; enzyme(s), e.g. chymosin or gastric lipase; enzyme inhibitor(s), e.g. tissue inhibitor of metalloproteinase (TIMP); hormone(s), e.g. growth hormone; lymphokine(s), e.g. an interferon; cytokine(s), e.g. interleukin-2, IL-4, IL-6 etc; 5 chemokine(s) eg macrophage inflammatory protein-2; plasminogen activator(s), e.g. tissue plasminogen activator (tPA) or prourokinase; or a natural, modified or chimeric immunoglobulin or a fragment thereof including chimeric immunoglobulins having dual activity such as antibody-enzyme or antibody-toxin chimeras.

- 10 In a highly preferred form of the invention the heterologous DNA sequence encodes either one or more of the ZP proteins from the animal species for which the CMV virus is specific or a viral antigen such as HA from influenza virus.

Zona pellucida is an extracellular matrix that surrounds growing oocytes and ovulated eggs. Antibodies directed against these proteins have been shown to 15 exert a contraceptive effect in some animals. For example, if MCMV is used as the viral vector into which the cassette is inserted, the heterologous DNA sequence may encode one or more of the murine ZP1, ZP2 or ZP3 proteins. Desirable the DNA sequence encodes ZP3 where immunocontraception of mice is required.

- 20 While the heterologous DNA sequence may encode only a single polypeptide sequence in the cassette, it will be appreciated by those skilled in the art that a plurality of heterologous DNA sequences may be linked together and inserted into the cassette to produce a plurality of polypeptides. Such polypeptides may be either produced as a fusion protein or engineered in such a manner to result in 25 two separate polypeptide sequences. Where the polypeptides are fused, at least one of the polypeptide sequences is preferably capable of being membrane bound. In such circumstances the polypeptide is preferably linked to a polypeptide which is expressed on a cells surface. For example, the polypeptide may be a transferrin receptor (eg. The human transferrin receptor) or any other 30 polypeptide sequence that comes with a natural membrane bound anchoring sequence. For example both haemagglutinin and ZP-3 come with their own anchoring sequences.

Multiple expression of polypeptides may also be useful for example if the CMV cassette was engineered to express a plurality of antigens (ie. protective antigens) along with cytokines or other immunomodulators to enhance the generation of an immune response. Alternatively, if the expression vector was used to deliver
5 immunogenic polypeptides to a host to provide a therapeutic effect against a particular type of viral infection, the cassette may be used to encode the immunogenic polypeptides in association with a range of epitopes which contribute to T-cell activity. In such circumstances the cassette preferably encodes epitopes capable of eliciting either a T helper cell response or a cytotoxic
10 T-cell (CTL) response or both.

In addition to the above, the heterologous DNA sequence may also encode one or more proteins which serve to enhance the effect of the protein being expressed. For example, ubiquitination of viral proteins expressed from DNA vectors results in enhancement of cytotoxic T-lymphocyte induction and antiviral protection after
15 immunisation. Thus, in a preferred embodiment of the invention the heterologous genetic sequence may encode ubiquitin in association with the protein to be expressed thus targeting the resulting fusion protein to proteosomes for efficient processing and uptake by the MHC class I complexes.

Infection by CMV often results in the down regulation of cellular MHC class I
20 heavy chains. Down regulation in this context, relates to the reduction in synthesis, stability or surface expression of MHC class I heavy chains. Recent studies have shown that *IE* polypeptides are more efficiently presented by interferon gamma treated HCMV-infected cells, than by untreated infected cells. Interferon gamma causes increased surface expression of MHC class I proteins.
25 Thus, increasing the expression of class I heavy chains in CMV-infected cells is preferred for the efficient generation of polypeptide-*IE*-specific CTLs. Therefore in a preferred form of the invention those genes capable of down regulating the major histocompatibility complex in CMV are preferably deleted from the recombinant virus. This may be achieved using the method described in US
30 Patent specification US 5720957 incorporated herein by reference [ii]. This patent specification teaches a method for identifying a gene sequence capable of down

regulating the major histocompatibility complex and deleting the identified gene sequence from the cytomegalovirus genome.

The present invention also provides a prophylactic or therapeutic substance that comprises a recombinant virus vector produced according to the present invention
5 in a pharmaceutically acceptable vehicle. In a preferred form of the invention the pharmaceutically acceptable vehicle may also comprise one or more excipients, adjuvants, stabiliser or other like substances. A pharmaceutical of this type should be prepared and used according to standard techniques known in the art.

Preferably the prophylactic or therapeutic substance comprises a
10 pharmaceutically acceptable excipient and an effective immunising amount of a recombinant CMV prepared according to the present invention. An effective immunising amount of the recombinant CMV may be determined according to techniques well known in the art.

The present invention also provides a method for manufacturing a prophylactic or
15 therapeutic substance, comprising the steps of:

- a) growing the recombinant CMV as herein described in a suitable host cell system; and
- b) mixing the resulting virus in an effective immunising amount with a pharmaceutically acceptable excipient.

20 Host cell lines contemplated to be useful in the method of the invention include any eucaryotic cell lines that can be immortalised and which is capable of supporting CMV replication, ie. are viable for multiple passages, (eg., greater than 50 generations), without significant reduction in growth rate or protein production. Useful cell line should also be easy to transfect, be capable of stably maintaining
25 foreign RNA with an unarranged sequence, and have the necessary cellular components for efficient transcription, translation, post-translation modification, and secretion of the protein. Currently preferred cells are those having simple media component requirements, and which can be adapted for suspension culturing. Most preferred are mammalian cell lines that can be adapted to growth
30 in low serum or serum-free medium. Representative host cell lines include human fibroblast (for human cells) or fibroblast cells derived the species for which the

CMV vector is specific, immortal NIH3T3 cells, E6/E7 immortalised cells, astrocytoma cell lines such as U373-MG, stromal cells and macrophage like cell lines and the like. Other useful cells and cell lines can be obtained from the American Type Culture Collection (ATCC), Rockville, Md. or from the European
5 Collection of Animal Cell Cultures, Porton Down, Salisbury SP40JG, U.K.

With respect to the transfection process used in the practice of the invention, all means for introducing viral vectors into a cell are contemplated including, without limitation, CaPO.sub.4 co-precipitation, electroporation, DEAE-dextran mediated uptake, protoplast fusion, microinjection and lipofusion. Moreover, the invention
10 contemplates either simultaneous or sequential transfection of the host cell with vectors containing the RNA sequences to be integrated into the genome.

Recombinant CMV may be purified by any protein purification method known in the field. Purification may be achieved by techniques such as, for example, salt fractionation, chromatography on ion exchange resins, affinity chromatography,
15 centrifugation, and the like. See, for example, Methods in Enzymology or Current Protocols in Protein Chemistry for a variety of methods for purifying proteins [iii][iv]. Preferably they are purified by a combination of sucrose and caesium chloride gradient centrifugation using methods which are well described in the literature.

20 Methods to administer CMV particles to uninfected individuals or to infected patients are well known to those of ordinary skill in the art. The method of choice to produce the most effective response will however need to be determined empirically and the methods described below are given as examples and do not limit the method of delivery.

25 Typically, therapeutics are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the particles encapsulated in liposomes.

Preferably the virus particles are formulated into therapeutics with excipients
30 which are pharmaceutically acceptable and compatible with the active ingredient.

Examples of excipients which may be used in such a formulation include, water, saline, ethanol, dextrose glycerol, or the like and combinations thereof. Further, if desired, the virus-like particle formulation may also contain minor amounts of auxiliary substances such as adjuvants, wetting, pH buffering agents, or
5 emulsifying agents that enhance the effectiveness of the vaccine. Suitable adjuvants which may be include in such formulations for example, aluminium hydroxide, N-acetyl-muramy1-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramy1-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)
10 methylamine (MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS).

Virus particles may also be formulated into therapeutics as neutral or salt forms. Pharmaceutically acceptable salts include, for example, the acid addition salts
15 (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with the free carboxyl groups may also be derived from in-organic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and
20 such organic bases as isopropylamins, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

Virus particle formulations may also be administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The quantity of virus particles to be administered will
25 generally depend on the regulatory elements included in the cassette and the half-life of the polyadenylation site. Moreover the dosage will also depend on the subject to be treated, the capacity of the subject's immune system to respond, and the degree of protection desired. Precise amounts of active ingredient required to be administered will depend on the judgment of the practitioner and
30 may be peculiar to each subject.

Formulations may be administered by the intradermal, subcutaneous or intramuscular routes, or by other routes including oral, aerosol, parenteral,

intravenous, intraperitoneal, rectal or vaginal administration. For example the virus-like particles may be administered parenterally, by injection, for example, either subcutaneously or intramuscularly. All the above formulations are commonly used in the pharmaceutical industry and are known to suitably qualified
5 practitioners.

In the case of oral administration, the virus particles may be delivered with diluents (water, saline etc) and/or delivery vehicles (tablets, capsules) which do not interfere with the activity of the particles. Oral formulations may include for example excipients such as, pharmaceutical grades of mannitol, lactose, starch,
10 magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. Such formulations may also take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders

Rectal or vaginal administration also requires specific formulation into acceptable forms that contain lubricants and or emulsifying agents. For example such
15 formulations usually include, traditional binders and carriers such as, polyalkylene glycols or triglycerides.

Further, the therapeutic may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of delivery may be with for example 1-10 separate doses, followed by
20 other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the need of the individual and be dependent upon the judgment of the practitioner.

25

Brief Description of Drawings

Further features of the present invention are more fully described in the following Figures and Examples. In the figures:

Figure 1 illustrates the plasmids and the cloning vectors used in the construction of recombinant MCMV expressing the genes mouse zona
30 pellucida 3, chicken ovalbumin (OVA) linked to the human transferrin cytoplasmic tail, influenza virus haemagglutinin from strain PR8 containing

the transmembrane binding region and the murine interleukin 6 gene. The plasmids containing the forementioned genes were all constructed in a similar way as described in the following examples, the difference being in the restriction enzyme sites used.

5 **Figure 2** illustrates plasmid pMV11 containing human CMV promoter and SV40 polyadenylation sequence and showing the multiple cloning sites.

Figure 3 illustrates the nucleotide sequence of the OVATfR construct cloned into pMV11.

10 **Figure 4** illustrates the sequence of MCMV *IE-2* region showing the location of HpaI restriction enzyme site into which the gene cassettes were inserted removing the TATA and AUG start region of *IE2*. A unique restriction site possibly suitable for insertion of the same cassette is also highlighted Nru I.

15 **Figure 5** illustrates plasmid pK181-H3L containing the 7.5 kb fragment of the MCMV K181 strain after digestion with the restriction enzyme HindIII and cloned into the promega plasmid pGEM11Zf.

Figure 6A illustrates restriction maps and salient features of completed plasmids containing OVATfR genes for the cotransfection and generation of recombinant MCMV.

20 **Figure 6B** illustrates restriction maps and salient features of completed plasmids containing ZP-3RJ genes for the cotransfection and generation of recombinant MCMV.

25 **Figure 7A(i)** illustrates restriction maps and salient features of completed plasmids containing a HA (PR8) gene for the cotransfection and generation of recombinant MCMV.

Figure 7A(ii) illustrates restriction maps and salient features of completed plasmids containing a mIL-6 gene for the cotransfection and generation of recombinant MCMV.

30 **Figure 7b** illustrates the process by which the recombinant MCMV is created and the constituent viral and vector constructs.

Figure 8 illustrates the replication of K181-OVATfR in the salivary gland of BALB/c mice.

Figure 9 illustrates the replication of K181- OVA/TfR in the visceral organs of BALB/c mice.

Figures 10(A-C) illustrates the replication of the recombinant MCMVs in tissue culture as measured by plaque assay.

5 **Figure 11** provide CTL assay results for BALB/c mice were inoculated with 2×10^4 plaque forming units (pfu) K181-OVA/TfR or K181-wild type (wt). Spleens were harvested at post infection re-stimulated in vitro and used in a CTL assay against K181-wt infected mouse embryo fibroblast (MEF) targets.

10 **Figure 12** provide CTL assay results for BALB/c mice were inoculated with 2×10^4 pfu K181-OVA/TfR or K181-wild type (wt). Spleens were harvested post infection re-stimulated in vitro and used in a CTL assay against K181-wt infected mouse embryo fibroblast (MEF) targets.

15 **Figure 13** provides a summary of the *in vivo* replication of recombinant MCMV containing different gene cassettes as described above in BALB/c mice ip-innolated with 2×10^4 pfu of tissue culture derived (MEF) recombinant virus.

20 **Figure 14** provides a summary table of the results obtained from ELISA assays conducted on serum obtained from BALB/c mice inoculated with 2×10^4 pfu tissue culture derived recombinant MCMV expressing membrane bound OVA, HA soluble and OVA with adjuvant or ultraviolet light inactivated (non-replicating) recombinant MCMV.

25 **Figure 15** provides a summary ELISA result of antibody isotype (IgG1 and IgG2a) titres to ZP-3 from animals inoculated with 2×10^4 pfu of recombinant MCMV-ZP3 virus 100 days previously.

Figures 16(A-C) shows the stimulation of a mucosal immune response in the salivary gland induced by recombinant MCMV expressing membrane bound OVA as measured by the ELISPOT assay.

30 **Figure 17** shows the stimulation of a mucosal immune response in the salivary gland induced by recombinant MCMV expressing membrane bound OVA as measured by the ELISPOT assay.

Figures 18(A-C) illustrates the replication of K181-OVA/TfR in interferon-receptor gene knock-out mice.

Figures 19(A-C) illustrates the replication of K181-OVA/TfR in interferon-alpha receptor gene knock-out mice.

Figure 20 provides a summary table demonstrating the replication of MCMV-IL6 (non-membrane antigen expression) and restoration of replication in the CMV organ of tropism for the virus in the salivary gland.

Figure 21 provides summary data and a graph for immunocontraception delivered by recombinant MCMV ZP3 in mice infected with 2×10^4 pfu virus in C57BL/6 mice.

Figure 22 provides summary data and a graph for immunocontraception delivered by recombinant MCMV ZP3 in mice infected with 2×10^4 pfu virus in BALB/c, mice.

Figure 23 provides summary data for immunocontraception delivered by recombinant MCMV ZP3 in mice infected with 2×10^4 pfu virus in outbred ARC/s mice.

Figure 24 provides summary data for immunocontraception delivered by recombinant MCMV-ZP3 in mice infected with 2×10^4 pfu virus in wild outbred mice.

Figure 25 provides summary data for immunocontraception in BALB/c mice infected with non-recombinant MCMV or MCMV-LacZ prior to infection with MCMV-ZP3.

Figure 26 provides a graph showing the titration of IL-6.

Figure 27 provides summary data for the protective effect of rMCMV-HA from lethal influenza virus infection 6 days after inoculation with rMCMV-HA.

Figure 28 provides summary data for the protective effect of rMCMV-HA from lethal influenza virus infection 21 days after inoculation with rMCMV-HA.

EXAMPLES

Further features of the present invention are more fully described in the following Examples. It is to be understood that the following Examples are included solely for the purposes of exemplifying the invention, and should not be understood in any way as a restriction on the broad description as set out above.

Cells. Primary mouse embryo fibroblast (MEF) cultures were prepared by trypsin dispersion of 15-day-old embryos from BALB/c mice [v], and were grown in minimal essential medium (MEM) containing 10% (v/v) newborn calf serum. MEF cultures were incubated at 37°C/5%CO₂.

- 5 **Virus.** K181 MCMV was the wild-type virus used throughout this study. The parental MCMV used for construction of the OVA recombinant MCMV was the RM427+ lacZ containing K181(smith)MCMV obtained from Prof. Ed. Mocarski, Stanford USA. This virus was constructed on the K181 (Smith) strain of MCMV and contains the lacZ gene, inserted into the Hpa I sites of the IE2 gene.
- 10 Construction and characterisation of RM427 recombinant has been described previously in Manning et al., 1992 [vi]. This virus contains the lacZ gene under transcriptional control of the human CMV immediate-early promoter-enhancer (positions -219 to -19 relative to the start site of transcription). RM427+ has been rescued for the sgg1 gene. The presence of sgg1+ has been confirmed by PCR.
- 15 All virus used in this study was propagated on MEF tissue culture in minimal essential medium supplemented with 10% Newborn Calf Serum.

Mice. 5-6 week old female BALB/c mice were obtained from Animal Resources Centre, Murdoch, Western Australia, Australia. Mice were kept under SPF conditions.

- 20 **Plasmid.** Plasmid cloning was by standard methods [vii]. All restriction enzymes were obtained from commercial suppliers and used according to manufacturers specifications, using the supplied buffers. A recombination plasmid designed to replace the lacZ gene in the HindIII L fragment of RM427+ with an OVA gene was constructed as described.
- 25 A pGEM11Zf(+) plasmid carrying the MCMV K181 *HindIII* L fragment in its HindIII site was obtained from Scalzo *et al* Department of Microbiology, University of Western Australia, Western Australia, Australia (see figure 1). This plasmid was designated pK181-H3L (see figure 5). A pBlueRIP-TfROVA plasmid containing the OVA gene with a transferrin receptor cytoplasmic tail sequence (TfR) fused
- 30 onto its amino terminus, was kindly donated by Prof. F.Carbone, Monash University, Melbourne, Victoria, Australia. Fusion of the TfR sequence to the OVA

gene ensures expression of OVA protein on the cell membrane of infected cell. The TfROVA sequence was excised from pBlueRIP-TfROVA with HindIII and blunt-ended by a fill-in reaction using Klenow DNA polymerase. The resulting fragment was blunt-end ligated into the SmaI site of pMV11 (see figure 2 & 3),
5 located between the human CMV IE1 promoter and a poly-adenylation stop sequence. The HindIII fragment, containing the regulatory elements and TfROVA, was again blunt-ended and inserted into the two HpaI sites of the HindIII L fragment on pK181-H3L, thereby removing the TATA box and transcriptional start site of IE2 gene [vi] (see figure 4). This plasmid was designated pK181-
10 H3L/MV11/OVA/TfR (see figure 6A).

Construction of plasmids containing alternative genes.

Plasmids containing murine ZP-3: (see figure 6B) Plasmid pUC19/ZP-3 containing the murine ZP-3 cDNA was obtained from Dr Ron Jackson VBCRC, Canberra, ACT, Australia. The cDNA coding for the ZP-3 was excised using the
15 restriction endonucleases Bam HI and Eco RI obtained from commercial suppliers. The cDNA was then ligated into the Bam HI/Eco RI site of the pMV11 vector and the DNA used to transform competent *E. coli* bacteria. After transformation, putative clones were screened and the clone was verified by standard molecular biological quality control methods. The expression cassette
20 containing the cDNA coding for ZP-3 was excised from pMV 11 using HindIII, the fragment blunt-ended using DNA polymerase (Klenow) and blunt-end ligated into the pK181-H3L plasmid. The pK181-H3L plasmid had first been digested to completion with the restriction enzyme HpaI the larger fragment purified by standard techniques of ethanol precipitation, agarose gel electrophoresis and gel
25 purification. Once again the ligation products were transformed into competent *E. coli* and putative clones verified by standard molecular biological techniques. The resultant plasmid DNA (pK 181-H3L containing ZP-3) was extracted, purified and used in the co-transfection reaction.

Plasmid pZP-3/1 containing the ZP-3 cDNA was obtained by extracting mRNA
30 from ovaries of BALB/c mice, random priming and DNA synthesis, the cDNA amplified using primers containing restriction sites and specific for the ZP-3 by PCR. The resultant DNA was cloned into pUC 19 and the DNA sequenced for

confirmation. The cDNA coding for the ZP-3 was excised using the restriction endonucleases Bam HI/Eco RI obtained from commercial suppliers. The cDNA was then ligated into the Bam HI/ Eco RI site of the pMV 11 vector and the DNA used to transform competent *E. coli* bacteria. After transformation, putative clones
5 were screened and the clone was verified by standard molecular biological quality control methods. The expression cassette containing the cDNA coding for ZP-3 was excised from pMV 11 using HindIII, the fragment blunt-ended using DNA polymerase (Klenow) and blunt-end ligated into the pK181-H3L, plasmid. The pK181-H3L plasmid had first been digested to completion with the restriction
10 enzyme HpaI the larger fragment purified by standard techniques of ethanol precipitation, agarose gel electrophoresis and gel purification. Once again the ligation products were transformed into competent *E. coli* and putative clones verified by standard molecular biological techniques. The resultant plasmid DNA (pK181-H3L containing ZP-3) was extracted, purified and used in the
15 co-transfection reaction.

Plasmid containing Influenza virus (PB8) haemagglutinin: (see figure 7A(i))
Plasmid pKG4 containing the Influenza virus (PR8) haemagglutinin cDNA was obtained from Dr. Bernadette Scott, University of Western Australia, Western Australia, Australia. The cDNA coding for the HA was excised using the
20 restriction endonucleases Bam HI and Bgl II obtained from commercial suppliers. The cDNA was then ligated into the Bam HI site of the pMV 11 vector and the DNA used to transform competent *E. coli* bacteria. After transformation, putative clones were screened and the clone was verified by standard molecular biological quality control methods. The expression cassette containing the cDNA coding for
25 HA was excised from pMV 11 using HindIII, the fragment blunt-ended using DNA polymerase (Klenow) and blunt-end ligated into the pK181-H3L plasmid. The pK181-H3L plasmid had first been digested to completion with the restriction enzyme HpaI, the larger fragment purified by standard techniques of ethanol precipitin, agarose gel electrophoresis and gel purification. Once again the ligation
30 products were transformed into competent *E. coli* and putative clones verified by standard molecular biological techniques. The resultant plasmid DNA (pK181-H3L containing HA) was extracted, purified and used in the co-transfection reaction.

Plasmid containing murine IL-6: (see figure 7A(ii)) Plasmid pCD-mIL6 containing the IL-6 cDNA was obtained from Dr Alistair Ramsay, Australian National University, Canberra, Australia. The cDNA coding for the IL-6 was excised using the restriction endonucleases Nla IV obtained from commercial suppliers. The cDNA was then ligated into the Sma I site of the pMV 11 vector and the DNA used to transform competent *E. coli* bacteria. After transformation, putative clones were screened and the clone was verified by standard molecular biological quality control methods. The expression cassette containing the cDNA coding for IL-6 was excised from pMV 11 using HindIII, the fragment blunt-ended using DNA polymerase (Klenow) and blunt-end ligated into the pK181-H3L plasmid. The pK181-H3L plasmid had first been digested to completion with the restriction enzyme HpaI, the larger fragment purified by standard techniques of ethanol precipitation, agarose gel electrophoresis and gel purification. Once again the ligation products were transformed into competent *E. coli* and putative clones verified by standard molecular biological techniques. The resultant plasmid-DNA (pK181-H3L containing IL-6) was extracted, purified and used in the co-transfection reaction.

Isolation of MCMV DNA.

Infectious MCMV DNA was prepared from RM427+ propagated in tissue culture and extracted from both supernatant and infected cells by protein kinase digestion, followed by phenol chloroform extraction and ethanol precipitation.

Construction of Recombinant Virus.

Recombinant MCMV was generated by cotransfecting 10-50µg of K181-RM427+ DNA and 2µg of HindIII linearised transfer plasmid, into MEF cells at 60-70% confluency. Cells were transfected by Calcium phosphate co-precipitation (Cellphect, Pharmacia) was allowed to proceed for 7hrs and followed with osmotic shock. The Transfected MEF monolayers were incubated for 4 days prior to sonication and the supernatant replated onto confluent fresh MEFs monolayers. The monolayer was overlaid with carboxymethylcellulose (CMC) during viral plaque development. After 6 days, potential recombinants were selected by their non-staining plaque phenotype after addition of X-gal. Half of the CMC overlay

was removed and replaced with X-gal CMC and the colour reaction allowed to proceed for 24hrs at 37°C. Clear plaques were picked and underwent two rounds of plaque purification as described in Manning and Mocarski (1988), to remove all contaminating parental RM427+[viii]. Confirmation of this was obtained by
5 screening MEF monolayers containing >1000 plaques with a non-viable X-gal stain. In short, MEFs were fixed in 0.5% glutaraldehyde, permeabilized, X-gal added in a $\text{Fe}^{2+}/\text{Fe}^{3+}$ containing solution and plaques were screened for blue colour. A high titre tissue-culture-propagated viral stock was subsequently prepared of the OVA recombinant MCMV.

- 10 The OVA-recombinant MCMV (rMCMV-TfROVA) was generated by cotransfection of RM427+ DNA with pH3L/MV11/TfROVA; the recombinant MCMV deleted for part of the *IE2* gene (rMCMV- \square /*IE2*) was generated by cotransfection of RM427+ DNA with pK181-H3L \square /*IE2*; the *lacZ*-containing revertant (rMCMV-TfROVA-REV) was generated by cotransfection of rMCMV-TfROVA DNA with pON427. This is
15 schematically represented in Figure 7B.

PCR. Following the immunostaining process, putative recombinant viral plaques were scrapped into TE containing 1% sarkosyl and 100 μ g/ml proteinase K, incubated at 56°C for 2hrs, phenol/chloroform extracted and the DNA ethanol precipitated. This DNA was resuspended in ddH₂O and used in a PCR reaction
20 to confirm the presence of the expected sized insert in the *IE2* region. Primers were designed which flanked the cloning site of the viral *IE2* gene. Forward primer Im2AF: CATTA AAACTATTGGTTCTA and reverse primer Im2AR: CCCATAGCCGAGCCCAATGCA. Expected sizes were 4.1kb for *lacZ* containing virus, 2.6kb for the recombinant MCMV clone containing the TfROVA construct
25 and a difference of 79bp for the \square /*IE2* construct when compared to wild type MCMV.

Primers used to confirm intact *sgg1* gene were as follows; forward primer H3JSG1F: ACAAGAGTCTGTCCGACCAC and reverse primer H3JSG2R: GCGGTACGTATACTGCCGTTA which amplify a 843bp fragment within the H3J
30 fragment of MCMV spanning the potential 323bp deletion of RM427. For all PCR reactions the Geneamp kit (Perkin Elmer) was used as per manufacturers

instructions. The template was amplified for 30 cycles: 94°C, 30sec; 52°C, 30sec; 72°C, 45sec in a 2400 PCR machine and products resolved by 0.9% AGE.

RFLP analysis and Southern blot analysis. Viral DNA was digested for 3hrs with restriction endonuclease *HindIII* and the fragments were resolved by pulsed
5 field electrophoresis on a 1.0% gel (CHEF; BioRad). Following electrophoresis, DNA fragments were transferred onto nylon membranes (Hybond N; Amersham) and probed as described in Manniatis et al., 1989 [i]. Probes were generated by PCR of the *IE1* and *IE2* genes, using the above-mentioned PCR primers. The OVA-specific probe was generated by excising the OVA fragment from pBlueRIP-
10 TfROVA. Probes were labelled with ³²P via random primer synthesis.

In vitro growth of recombinant MCMV. MEF cells were infected at a multiplicity of infection of 5 with rMCMV-TfROVA, RM427+, rMCMV-TfROVA-REV, rMCMV-
□IE2, K181-HA, K181-HA_REV and K181 MCMVs. After the initial 1hr incubation, the cell monolayers were washed with citric acid buffer of pH 3.0
15 containing 40mM citric acid, 10 mM KCl and 135 mM NaCl, to remove reversibly bound virus particles. At specified hours post-infection, virus was harvested from the monolayer and the supernatant, sonicated, and virus titres quantitated by plaque assay on MEF cells. The limit of detection was 10 pfu/ml for each monolayer assayed. The results are illustrated in Figures 10(a), (b) and (c).

20 **Generation of hyperimmune sera against OVA, HA, ZP-3 and MCMV.**

Adult BALB/c mice were inoculated i.p. with OVA-adjuvant emulsion, and given two consecutive boosters two weeks apart. Blood was collected by exsanguination (heart puncture) and allowed to clot at room temperature for 1 hour prior to centrifugation for 1 min. Sera were stored at -20°C until use.

25 Similar experiments were performed for the generation of hyperimmune sera against ZP-3, and HA. The hyperimmune sera for HA was raised from mice infected with influenza PR8 with the hyperimmune sera for ZP-3 was raised against the purified B-cell epitope protein in ZP-3.

Detection of expression of OVA by recombinant MCMV.

The expression of OVA by recombinant virus was measured by immunostaining viral plaques infected with the recombinant virus using hyperimmune serum raised in mice against the purified OVA protein.

- 5 Expression of OVA by rMCMV-TfROVA was confirmed *in vitro* through immunostaining, using anti-OVA hyperimmune serum. Virus infected cells were fixed with 50/50% methanol/acetone and underwent a blocking step with normal mouse serum, prior to the addition of primary antibody in phosphate-buffered saline with an osmolarity adjusted to be equivalent to that of mouse (MOBS).
- 10 After a 37°C incubation for 1hr, the primary antibody was removed, the monolayer washed with MOBS, and secondary alkaline phosphatase-conjugated goat anti-mouse antibody added and incubated as before. Substrate was added and the presence of stained plaques observed and recorded.

- The enzyme-linked immunosorbent assay (ELISA) used was a modification of the
- 15 method described in [ix]. For the detection of anti-MCMV antibodies, plates were coated with MCMV antigen, prepared by infecting MEF monolayers with MCMV until near 100% CPE observed, clarifying the culture fluid by centrifugation at 4500g/20min/4°C and then ultracentrifugation at 30000g/2hrs/4°C [i]. Viral pellets were resuspended in MOBS and the antigen titrated for optimum dilution. Antigen
 - 20 was coated onto the plates in carbonate/bi-carbonate buffer pH 9.6, overnight at 4°C. Primary antibody was added at a 1/10 dilution and consecutive doubling dilutions of sera were made in MOBS containing 10% bovine serum albumin (BSA; CSL) and 0.05% Tween 20 (Sigma). The secondary antibodies used were biotinylated goat anti-mouse anti-IgM, IgG1 or IgG2a (Southern Biotechnology,
 - 25 Birmingham, USA). Streptavidin alkaline phosphatase (Pierce, USA) was added in an additional step. The substrate solution contained 5 mg p-Nitrophenyl Phosphate tablets (Sigma) in 5 ml diethanolamine buffer pH 9.8. The reactions were read after 20 min at 405 nm using a BioRad Model 3550 Microplate reader (BioRad, Hercules, California, USA). All incubation steps were performed for 1
 - 30 hour at 37°C. Absorbance values (y-axis) were plotted against the log of the dilution factors (x-axis). Serum titre is given as the preceding value on the x-axis

of the intersect of the linear region of the curve with NMS + 3 standard deviations and expressed as reciprocal dilutions. ELISAs for the detection of anti-OVA or anti- β -galactosidase antibodies were performed as described above, using plates coated with either 1.0 μ g/ml OVA (Grade VI; Sigma) or 0.5 μ g/ml β -galactosidase, respectively.

Immunofluorescence microscopy. MEF monolayers on 8-well glass microscope slides (LAB-TEK, Nalge Nunc International) were infected for 4h with rMCMV-TfR/OVA or K181-WT, washed in MOBS/1%FCS and fixed with methanol/acetone (50%/50%) for 2 mins. Fixed cells were washed in MOBS/1%FCS, blocked in 10% normal mouse serum/MOBS/1%FCS for 30 min at room temperature and again washed in MOBS/1%FCS. This was followed with a 45 min incubation at RT with the primary antibody, rabbit anti-OVA polyclonal (ICN Biomedicals, Inc.) diluted 1/100 in MOBS/1%FCS. Monolayers were washed in MOBS/1%FCS again and incubated with anti-rabbit immunoglobulin biotin conjugated (Silenus) at 1/200 dilution in MOBS/1%FCS for 1hr at RT. A third incubation step of 1hr at RT with Fluorolink Cy3 labelled streptavidin (LIFESCIENCE) at 1/1000 dilution in MOBS/1%FCS was followed with a MOBS/1%FCS wash and mounted in MOBS/50%glycerol. Monolayers were examined by immunofluorescent microscopy with a Leitz Wetzlar microscope.

Detection of expression of expression of ZP-3 by recombinant MCMV: The expression of ZP-3 was measured by immunostaining viral plaques infected with the recombinant virus using hyperimmune serum raised in mice against the purified B-cell epitope.

Detection of expression of expression of HA by recombinant MCMV: The expression of Influenza virus HA was measured by immunostaining viral plaques infected with the recombinant virus using hyperimmune serum raised in mice infected with influenza virus PR8

Detection of expression of expression of IL-6 by recombinant MCMV: Expression of the IL-6 was measured in a bioassay whereby indicator cells dependent on the presence of IL-6 were fed serial dilutions of cell culture

supernatants that had been infected with the IL-6 containing recombinant MCMV. This is illustrated in Figure 26.

***In vivo* growth of recombinant MCMV.**

- 5 Three week-old female BALB/c mice were inoculated intraperitoneally (ip) with 2×10^4 pfu of tissue-culture-propagated (TCV) MCMV. At days 3, 7, 14, 28 and 42-post infection, three mice per time point were sacrificed and salivary glands, spleens and livers harvested. Similarly, IFN α/β and γ receptor knockout mice (129Sv(Ev)) and control mice were inoculated with 2×10^4 pfu of TCV MCMV.
- 10 Individual organs were weighed prior to homogenisation in 2ml MEM + 2% NCS, centrifuged at 2000g for 20 min at 4°C and stored at -70°C until use. Plaque assays were performed on MEF using four-fold serial dilutions of these homogenates, as described in [x]. Cultures were overlaid with 1.75% methylcellulose (Sigma) and left for four days at 37°C/5%CO₂. Plaques were
- 15 enumerated after 24-hr staining in 0.5% (w/v) methylene blue containing 10% formaldehyde. Organ virus titres were expressed as pfu/g organ. The limits of viral detection in these assays were 200 pfu/g salivary gland or spleen and 20pfu/g liver.

- RM427+ used as the control. Plaque assays were described in Scalzo et al 1990
- 20 [xi].

- In BALB/c mice inoculated with 2×10^4 pfu TCV, replicating K181-OVA/TfR was not detected in the salivary gland, which is the organ of persistence for MCMV (see figure 8). Virus was only detected using the following PCR experiment. However, K181-OVA/TfR did replicate in visceral organs at levels comparable to parental
- 25 RM427+MCMV during early infection times following i.p. inoculation with a higher dose (5×10^4 pfu TCV) (see figure 9). K181-OVA/TfR was still not detected by plaque assay in the salivary glands of BALB/c mice given 10^6 pfu TCV ip. 5×10^4 pfu ip for *in vivo* replication in the liver and spleen, organs collected at days 3 and 5 post infection.
- 30 BALB/c mice were inoculated with 2×10^4 pfu K181-OVA/TfR or K181-wild type (wt). Spleens were harvested post infection restimulated *in vitro* and used in a

CTL assay against K181-wt infected mouse embryo fibroblast (MEF) targets. The data from these experiments are shown in figures 11 and 12.

Figure 13 provides a summary of the *in vivo* replication of recombinant MCMV containing different gene cassettes as described above in BALB/c mice ip
5 inoculated with 2×10^4 pfu of tissue culture derived (MEF) recombinant virus.

ELISA For the detection of anti-MCMV antibodies, plates coated with MCMV antigen prepared by infection of MEF monolayers and at 90% CPE, the culture fluid clarified by centrifugation at 3000RPM and then ultracentrifugation at 40000RPM, 3hrs. Viral pellets re-suspended in PBS and used to coat plates.
10 Primary antibody 1hr, wash, 2 antibody conjugate either IgM/IgG or anti-IgG1 or IgG2a used. Serum titre given as intersect of linear region of curve with NMS+3SD and expressed as reciprocal dilutions [i].

Either anti OVA or anti β -galactosidase measured. Plated coated with 1.0 or 0.5 mg/ml protein respectively in alkaline phosphate buffer.

15 Figure 14 provides a summary table of the results obtained from ELISA assays conducted on serum obtained from BALB/c mice inoculated with 2×10^4 pfu tissue culture derived recombinant MCMV expressing membrane bound OVA, HA and soluble OVA with adjuvant or ultraviolet light inactivated (non-replicating) recombinant MCMV. The recombinant MCMV OVA or HA stimulates an immune
20 response to the heterologous antigen of a predominantly IgG2a isotype when compared to administration of the antigen by other means and indicates an ability to switch the antibody response from what otherwise occurs. Serum boosting of the IgG2a response occurs after a second (boost) with MCMV expressing HA.

Figure 15 provides a summary ELISA result of antibody isotype (IgG1 and IgG2a)
25 titres to ZP-3 from animals inoculated with 2×10^4 pfu of recombinant MCMV-ZP3 virus 100 days previously.

Figures 16 and 17 show the stimulation of a mucosal immune response in the salivary gland induced by recombinant MCMV expressing membrane bound OVA as measured by the ELISPOT assay. Antibody secreting cells from the cervical
30 lymph nodes of BALB/c mice were infected with 2×10^4 rMCMV-OVA ip. IgA

secreting cells were detected by this method and not by inoculation with OVA/adjuvant.

Figure 18(A), (B) and (C) illustrate the replication of K181-OVA/TfR in interferon-receptor gene knock-out mice. K181-OVA TfR replication in IFN-gamma R gene-
5 knock out (gko) mice is not significantly different to that obtained in parental 129/Ev/Sv mice, however, it appears that IFN-alpha does have a role in controlling viral replication in the spleen. Loss of both IFN-alpha and IFN-gamma function significantly increases virus replication in the visceral organs and demonstrates that K181-OVA/TfR is not defective in salivary gland replication.
10 Mice were inoculated with 2×10^4 pfu TCV ip.

Figures 19 (A), (B) and (C) illustrate the replication of K181-OVA/TfR in interferon-alpha receptor gene knock-out mice. In comparison to the results present in figure 18, a dose dependent effect is observed in 129/Ev/Sv control mice, inoculated with 2×10^5 pfu TCV, where K181-OVA/TfR replication is detected at
15 higher levels in the visceral organs and also in the salivary gland. IFN-alpha plays a role in controlling K181-OVA/TfR replication in these organs. The parental RM427+ replication in IFN-alpha R gko mice was increase by the same order of magnitude.

Figure 20 provides a summary table demonstrating the replication of MCMV-IL6
20 (non-membrane antigen expression) and restoration of replication in the CMV organ of tropism for the virus in the salivary gland.

In the following experiments BALB/c, C57/BL6 and ARC female mice at 6-8 weeks of age were inoculated ip. 2×10^4 recombinant virus containing the gene encoding ZP-3. Males were added 21 days post infection in the following ratios:

- 25 Uninfected, 1 male + 3 females, 3 groups. (9 females)
 RM427+, 1 male + 3 females, 3 groups. (9 females)
 rMCMV-mZP3, 1 male + 3 females, 3 groups. (9 females)

Pregnancies were then measured. No pups born to 9 rMCMV-ZP3 infected
30 female BALB/c mice after 90 days mating. In contrast 141 pups were born to 9 control females. Decrease in the number of pups born to rMCMV-ZP3 infected female C57BL/6 mice compared to controls. Marked depletion of mature ovarian

follicles in BALB/c and C57BL/6 first observed at day 7 post infection, resolving in C57BL/6 by day 35 post infection. Seroconversion to MCMV by 1 of 3 male CBA mice housed with rMCMV-ZP3 infected female mice.

Figure 21 provides summary data and a graph for immunocontraception delivered
5 by recombinant MCMV ZP3 in mice infected with 2×10^4 pfu virus in C57BL/6 mice.

Figure 22 provides summary data and a graph for immunocontraception delivered by recombinant MCMV ZP3 in mice infected with 2×10^4 pfu virus in BALB/c, mice.

Figure 23 provides summary data for immunocontraception delivered by recombinant MCMV ZP3 in mice infected with 2×10^4 pfu virus in outbred ARC/s
10 mice.

Figure 24 provides summary data for immunocontraception delivered by recombinant MCMV ZP3 in mice infected with 2×10^4 pfu virus in wild outbred mice.

Mice inoculated with wild parent MCMV and boosted twice with MCMV-ZP3 is
15 shown in figure 25. This figure illustrates the immunocontraceptive effect in wild outbred mice.

Groups of mice were vaccinated with MCMV and subsequently challenged with a lethal dose of live influenza virus either 6 days or 21 days post infection. The results of these experiments highlight the protective effect of the recombinant
20 McMV-HA (see figures 27 & 28).

Alternate method for insertion of Heterologous DNA into CMV vector

To develop a unique method for the insertion of heterologous antigens into the MCMV genome a desired gene of interest or antigenic sequence is cloned into a transfer vector such that it is flanked by the bacterial transposable element
25 sequences, Tn7L and Tn7R.

A bacterial plasmid (pCMH151) has been constructed by standard cloning techniques that contains a bacterial origin of replication, a kanamycin resistance (Km^R) marker gene, and a lacZ gene that contains the attachment sequence (att:Tn7) for the bacterial transposable element, Tn7. This plasmid has

subsequently been digested with the appropriate restriction enzyme (*NotI*) and inserted into a second plasmid (pK181H3L-*NotI*) that is resistant to ampicillin (Amp^R) and has been engineered to contain a *NotI* restriction site within the *IE2* gene of the *HindIII* L fragment (previously a *HpaI* site). The resulting clone
5 (pK181H3L/pCMH151) contains the elements of pCMH151 flanked by MCMV sequences that are homologous to the *IE1* and *IE2* regions of the MCMV genome.

This transfer vector can be transformed into bacterial cells that contain the MCMV genome that has been cloned as a bacterial artificial chromosome (BAC). In the presence of bacterial transposition proteins supplied by a helper plasmid, the
10 desired antigenic sequence in the transfer vector can be transposed into a specific attachment site (*att:Tn7*) that has been engineered into the MCMV BAC plasmid. Positive MCMV BAC plasmids containing the inserted antigenic sequence is identified by disruption of a *lacZ* gene sequence associated with the *att:Tn7* site.

15 Following characterisation of the clones by standard techniques, the MCMV BAC plasmids can then be purified and transfected onto eukaryotic cells to recover infectious viral progeny containing the foreign antigenic sequence.

To confirm that it is possible to transpose a genetic element into the *att:Tn7* site of the engineered plasmid pK181H3L/pCMH151, a model for the *in vitro*
20 transposition procedure was developed. Bacterial cells (DH10B) were transformed with the plasmid pK181H3L/pCMH151. Positive transformants were identified by their brilliant blue colour on L-agar plates containing X-gal, kanamycin and ampicillin. A positive transformed colony was grown in culture and subsequently these cells were transformed with the plasmid pMON7124, a
25 helper plasmid containing the genes for the bacterial transposition proteins and a tetracycline (Tc^R) resistance marker.

To demonstrate transposition, a transfer vector, pFASTBAC, containing a gentamycin resistance marker (Gm^R) flanked by the transposable element sequences *Tn7R* and *Tn7L* was transformed into the host bacteria containing
30 pK181H3L/pCMH151 and pMON7124. It was observed that several white colonies grew on L-agar plates containing Km, Gm, Amp and Tc, indicating that

the transposable element from pFASTBAC had inserted into pK181H3L/pCMH151, disrupting the lacZ gene. No colonies were observed when the host bacteria alone were plated onto L-agar plates containing Km, Gm, Amp and Tc.

- 5 These results indicated that transposition of the Gm^R gene had occurred into the specific attachment site of pK181H3L/pCMH151 demonstrating that it is possible to transpose desired sequences into a specific attachment site.

Those skilled in the art will appreciate that the invention described above is susceptible to variations and modifications other than those specifically described.

- 10 It is to be understood that the invention includes all such variation and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in the specification, individually or collectively, and any and all combinations or any two or more of the steps or features.

15

REFERENCES

- i. Sambrook J., Fritsch, E.F. and Maniatis T., (1989). In: "Molecular Cloning: A Laboratory Manual", 2nd Edition. [Cold Spring Harbour, N.Y]
- ii. US 5720957 (in the name of American Cyanamid Company)
- iii. Methods in Enzymology. (1955–1999) [Academic Press .N.Y]
- 20 iv. Current Protocols in Protein Chemistry
- v. Chalmer J.E., Mackenzie J.S. and Stanley N.F. (1977). Resistance to murine cytomegalovirus linked to the major histocompatibility complex of the mouse. *The Journal of General Virology*, **37**: 107-114
- vi. Manning W.C., Stoddart C.A., Lagenaur L.A., Abenes G.B. and Morris E.S.
- 25 (1992) Cytomegalovirus determinant of replication in salivary glands. *Journal of Virology*. **66**:3794-3802.
- vii. Sambrook J., Fritsch, E.F. and Maniatis T., (1989). In: "Molecular Cloning: A Laboratory Manual", 2nd Edition. [Cold Spring Harbour, N.Y]
- viii. Manning W.C. and Mocarski E.S. (1988). Insertional mutagenesis of the
- 30 murine cytomegalovirus genome: one prominent alpha gene (*IE2*) is dispensable for growth. *Virology*. **167**: 477-84.

- ix. Lawson, C.M., Grundy J.E. and Shellam G.R. (1988). Antibody responses to murine cytomegalovirus in genetically resistant and susceptible strains of mice. *The Journal of General Virology*. **69**:1987-1998.
- 5 x. Lawson, C.M., Grundy J.E. and Shellam G.R. (1987). Delayed-type hypersensitivity responses to murine cytomegalovirus in genetically resistant and susceptible strains of mice. *The Journal of General Virology*. **68**: 2379-2388.
- 10 xi. Scalzo A.A., Fitzgerald N.A., Simmons A., La Vista A.B. and Shellam G.R. (1990). CMV-1, A genetic locus that controls murine cytomegalovirus replication in the spleen. *The Journal of Experimental Medicine*, **171**: 1469-1483.

The claims defining the invention are as follows:

1. A recombinant Cytomegalovirus comprising: a Cytomegalovirus genome including within the immediate-early (*IE*) gene region of the virus genome, a genetic cassette comprising at least a promoter adjacent to at least a DNA
5 sequence and a polyadenylation stop sequence, wherein (1) the promoter is adapted to drive the expression of the DNA sequence, and (2) the promoter, DNA sequence and the polyadenylation stop sequence are heterologous to the virus in which the cassette is inserted.
- 10 2. A recombinant Cytomegalovirus according to claim 1 wherein the genetic cassette is inserted within the *IE* region in such a manner that it inactivates at least a gene which encodes for an *IE* protein.
- 15 3. A recombinant Cytomegalovirus according to claim 1 or 2 wherein the genetic cassette contains an identifiable phenotype.
4. A recombinant Cytomegalovirus according to claim 3 wherein insertion of the cassette into the viral genome leads either to activation of the identifiable phenotype or its deletion from the viral genome therein providing a means
20 for selecting for the transformed virus.
5. A recombinant Cytomegalovirus according to any one of claims 1 to 4 wherein the genetic cassette is inserted into the Cytomegalovirus genome within or between the *IE* genes in the selected Cytomegalovirus.
25
6. A recombinant Cytomegalovirus according to claim 5 wherein the genetic cassette is inserted between genes *IE*-1 and *IE*-2.
7. A recombinant Cytomegalovirus according to claim 5 wherein the genetic
30 cassette is inserted after the *IE*-2 gene.
8. A recombinant Cytomegalovirus according to any one of claims 1 to 7 wherein the promoter is a viral promoter sequence.

9. A recombinant Cytomegalovirus according to any one of claims 1 to 7 wherein the promoter is a mammalian promoter sequence.
- 5 10. A recombinant Cytomegalovirus according to any one of claims 1 to 7 wherein the promoter is a bacterial promoter sequence.
11. A recombinant Cytomegalovirus according to any one of claims 1 to 7 wherein the promoter is a bacteriophage promoter sequence.
- 10 12. A recombinant Cytomegalovirus comprising: a Cytomegalovirus into which is inserted within the (*IE*) gene region of the virus genome, genetic cassette contains at least a HCMV *IE*-1 promoter adjacent to at least a DNA sequence and a polyadenylation stop sequence, wherein (1) the HCMV *IE*-1 promoter is adapted to drive the expression of the DNA sequence, and (2)
- 15 both the DNA sequence and the polyadenylation stop sequence are heterologous to the virus.
13. A recombinant Cytomegalovirus according to any one of claims 1 to 12 wherein the polyadenylation stop sequence is derived from the SV40 polyadenylation stop sequence.
- 20 14. A recombinant Cytomegalovirus according to any one of claims 1 to 12 wherein the polyadenylation stop sequence is derived from human or bovine growth factor.
- 25 15. A recombinant Cytomegalovirus according to any one of claims 1 to 12 wherein the polyadenylation stop sequence is derived from bovine growth factor p[A].
- 30 16. A recombinant Cytomegalovirus according to any one of claims 1 to 12 wherein the polyadenylation stop sequence is derived from retroviral 3'LTR.

17. A recombinant Cytomegalovirus according to any one of claims 1 to 16 wherein the heterologous DNA sequence encodes part or all of any natural or recombinant protein that is heterologous to the Cytomegalovirus from which the recombinant virus is formed.
- 5
18. A recombinant Cytomegalovirus according to any one of claims 1 to 16 wherein the heterologous DNA sequence encodes zona pellucida proteins from the animal species for which the CMV virus is specific.
- 10
19. A recombinant Cytomegalovirus according to any one of claims 1 to 16 wherein the heterologous DNA sequence encodes a viral antigen such as HA from influenza virus.
20. A recombinant Cytomegalovirus according to any one of claims 1 to 16 wherein there is a plurality of heterologous DNA sequences inserted in such a way as to produce a plurality of polypeptides.
- 15
21. A recombinant Cytomegalovirus according to any one of claims 1 to 16 wherein the heterologous DNA sequence encodes epitopes capable of eliciting either a T helper cell response or a cytotoxic T-cell response or both.
- 20
22. A recombinant Cytomegalovirus according to any one of claims 1 to 16 wherein the heterologous DNA sequence encodes ubiquitin in association with the protein to be expressed.
- 25
23. A recombinant Cytomegalovirus according to claims 1 to 21 wherein those genes capable of down regulating the major histocompatibility complex in CMV are preferably deleted from the recombinant virus.
- 30
24. A prophylactic or therapeutic substance that comprises a recombinant virus vector according to any one of the preceding claims in a pharmaceutically acceptable vehicle.

25. A prophylactic or therapeutic substance according to claim 24 wherein the pharmaceutically acceptable vehicle comprises one or more excipients, adjuvants, stabiliser or other like substances.
- 5
26. A prophylactic or therapeutic substance according to claim 24 wherein the prophylactic or therapeutic substance comprises a pharmaceutically acceptable excipient and an effective immunising amount of a recombinant CMV prepared according to the present invention.
- 10
27. A method for manufacturing a prophylactic or therapeutic substance comprising the steps of:
- a) growing the recombinant CMV in a suitable host cell system; and
 - b) mixing the resulting virus in an effective immunising amount with a
- 15 pharmaceutically acceptable excipient.
28. A method according to claim 27 wherein the host cell line is a mammalian cell line that can be adapted to grow in low serum or serum-free medium.
- 20 29. A recombinant virus according to claim 1 substantially as herein described in the examples

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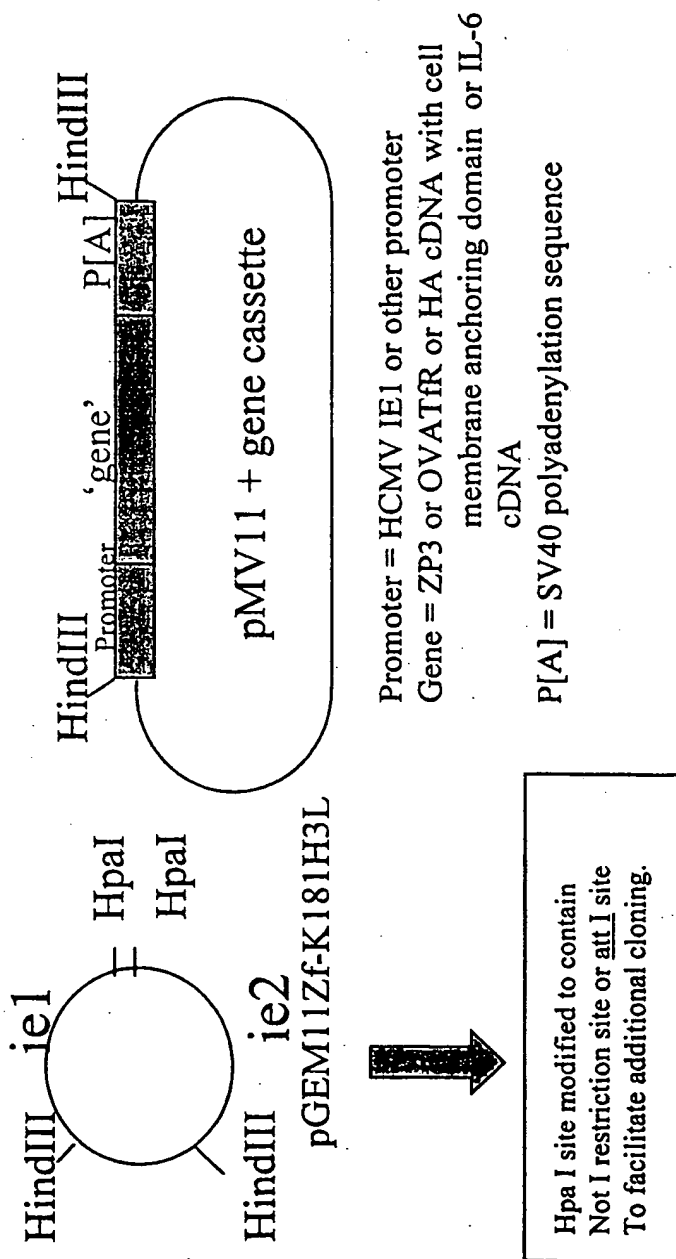
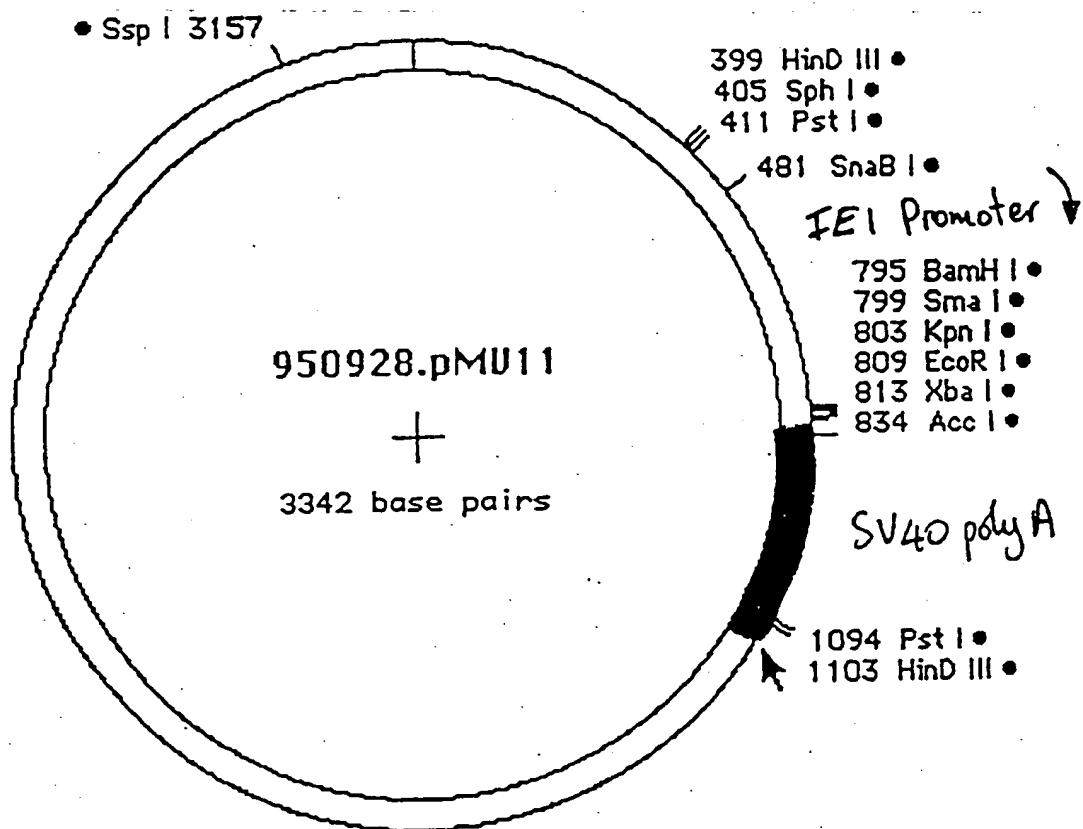


Fig. 1

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**Fig 2.**

	10	20	30	40	50	60	70	80
1	cctaaactta	gcaagcttga	attccatcgg	gtggcggttc	gggaaggagg	acgcgctagt	gttctcttgt	gtggcagttc 80
81	agaatgatgg	atcaagcttag	atcagcattc	tctaacttgt	ttggtggaga	accattgtca	tataccgggt	tcagcctggc 160
161	tcggcaagta	gatggcgata	acagtcattgt	ggagatgaaa	cttgctgtag	atgaagaaga	aaatgctgac	aataacacaa 240
241	aggccaatgt	cacaaaacca	aaaaggtgta	gtggaagtat	ctgctatggg	actattgctg	tgatcgtctt	tttcttgatt 320
321	ggattttatga	tggctactt	gggtctattgt	aaaggggtag	aaccaaaaac	tgagtgtgag	agactggcag	gaaccgagtc 400
401	tcagatgagg	gaggagccag	gagaggactt	ccgtgagat	caagccagag	agctcatcaa	ttcctgggta	gaaagtcaga 480
481	caaatggaa	tatcagaaat	gtccttcagc	caagctccgt	ggattctcaa	actgcaatgg	ttctggttaa	tgccattgtc 560
561	ttcaaggagc	tgtgggagaa	aacatttaag	gatgaagaca	cacaagcaat	gccttcaga	gtgactgagc	agaaaagcaa 640
641	acctgtgcag	atgatgtacc	agattggttt	atttagagtg	gcataaatgg	caactgagaa	aatgaagatc	ctggagcttc 720
721	catttgccag	tgggacaatg	agcatgttgg	tgcgtgtgcc	tgatgaagtc	tcaggccttg	agcagcttga	gagtataatc 800
801	aactttgaaa	aactgactga	atggaccagt	tctaattgta	tggaagagag	gaagatcaaa	gtgtacttac	ctcgcatgaa 880
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1041	gaggtggtag	ggtcagcaga	ggctggagtg	gatgctgcaa	gcgtctctga	agaatttagg	gctgaccatc	cattctcttt 1120
1121	ctgtatcaag	cacatcgcaa	ccaacgccgt	tcctctcttt	ggcagatgtg	tttcccttta	aaaagaagaa	agctgaanaa 1200
1201	ctctgtccct	tccaacaaga	ccaagagcac	tgtagtatca	gggttaaaat	gaaaagtatg	ttctctgctg	catccagact 1280
1281	tcataaaagc	tggagcttaa	tctagaanaa	aaatcagaaa	gaaattacac	tgtgagaaca	ggtgaatttc	acttttcctt 1360
1361	tacacagagt	aatactggta	actcatggat	gaaggcttaa	gggaatgaaa	tggactcac	agtactgagt	catcacactg 1440
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1601	caattgcttg	ttctctgtgt	tcctgatact	acaaggctct	tcctgacttc	ctaaagatgc	attataaaaa	tcttataatt 1680
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1761	cccatatgta	atgggtcttg	tgaatgtgct	cttttgttcc	tttaatcata	ataaaaacat	gtttaagcgg	aattccatgg 1840
1841	agatccaaag	tttaaatggt	aatgtgccc	tggacaggct	gaactttgag	ctaggagtag	ctattgacca	gtttttagta 1920
1921	gttttttgagg	atgtaaaagg	cactggaggg	gagtcacagag	atttgccctc	aggtcaggga	attataaacc	tggacaattt 2000
2001	aagggattat	ttggatggca	gtgttaagg	aaacttagaa	asgtccctac	agtcgtgtca	tgatacataat	cagccatacc 2080
2081	acattttag	aggttttact	tgttttaaaa	aaactccac	acctccccc	gaacctgaaa	cataaaatga	atgcaattgt 2160

HindIII restriction site, nt#s 13 and 1847 PstI restriction site, nt# 433

Nucleotide sequence of the OVA/TR cloned into plasmid MV11 (HindIII fragment)

Fig. 3

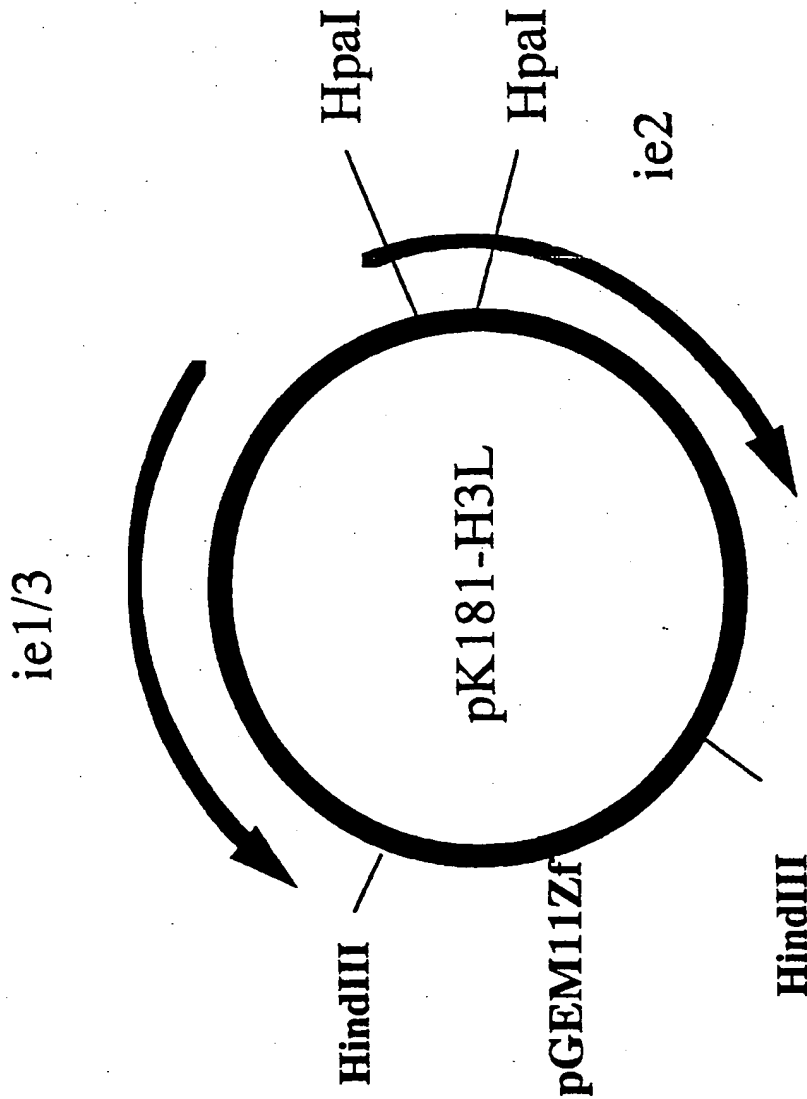
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9121	TGGGAAATGG	GGTAAATAAGT	CCCGTATTA	CTACATAGG	GGGCTTTGG	CTTTGCAAT	TAGGGATTT	CAGTGCATTT
9201	AGCATTAATA	ACTATTGGTT	CTAGTCATA	AACGGGCGGA	GTAAACCAT	TAAAGCTGT	CCCCATGCC	ATTCGAGCCC
9281	GGACAGGAG	GGACCGGGG	TCGATACGAC	CCATCTACG	TTAAGGAGG	GGTGAACGGA	GGCCCCAGG	CCGGGTAAGG
9361	TTGCATTGGG	CTGGGCTATG	GGACGGGGAT	TTGGCCCGGT	ACTTTAATAT	GGGTGGGGTC	TTATGGGTAG	GGGGCTATT
9441	AAATAAACGG	CCCTACCCCA	TACATATTGC	GTCAGTAAT	TAGAAGCCCC	CTACCAACGCC	ATTGGCGCCC	GCCCATAGCG
9521	GTACTTTTCGG	TTCTATACAA	GATGGCTACC	GCGGTTTCG	ATCGCTCCCG	GGGTCTCAT	TTCTATAGCT	GGAAGCATC
9601	CGACCCCCAT	GATCTGTGCC	TTAGACCCCA	GGGTACGTGC	CTGGATAGGT	GGGGACCCC	TAGCGGCTGC	TTGTATTTT
9681	ATATCAGAGA	TGAAATAATCA	CAGCGGCGCG	TAACAGCTGT	CAGGGCTATA	CAAGATGGCG	ACCGTACTTT	TCGATCGCTT
9761	TTCTTTCCCT	CGATCCTGCT	TCCGAAACGC	AACCCACCCC	CGSGGTATGT	GCCTTAGACC	CTATAGATGC	CCCCAGGAC
9841	GCAGGAAATA	TTTATAGCTCC	CCCTATATA	TTTATTCGAG	CGATCGAAAT	CCGCGGACCG	CCGGGTTT	TACTTCGTT
9921	TTCTCGGTCC	CCGCTCGGCT	CGGATTCGGA	GCAGAAATAG	GGCTAGAAAG	CTCGCTCAC	CTGCCGAAAC	GCGTTGTCG
10001	GATCATCCCG	GGAGGGCCAG	CGTAGTCTCC	GTTGCTGGGT	TGGCCGAGG	GATCGAGTCG	AGCGGCGCG	GGGACGCCG
10081	GAACAGAGAG	GGATCGGGAG	CGGCGGCGCG	GTTGGCTCGG	GAGCGGCGG	GAGACGGTCT	GCAGAGCTCC	AGAGCTGCGT
10161	GTCTGTGAC	TGAACAGTTG	GGAAATAATAC	TGGATCTTAT	AATGACCCAG	GGGAAGGGGT	CCGAGATCCT	CCAGTGTCC
10241	GAGAGTGCCA	AGAGACACTC	CTGTTGAAAA	GCAGCTCTAA	AACGTTTTC	TGCTCATCTC	TGCCAGACCC	CCGCCACTG
10321	ACAGAGCTCA	ATTGATAGCA	GATGCTCTCT	TTGGAAGGAC	ACAGCTTCC	ACAAACACCG	TTCTCTACTG	AGGTGCATG
10401	TCGGGAGGG	AAGTCACTCT	TCTTGTTCAT	TGTGGAGGTT	GTGGGCCCCA	GGCTCTGGAC	GACCTTGCCC	CGGGCTTCT
10481	TCCTCTGAT	CCAGGTAGAT	TGACGTGCGG	ACATCGGGA	CGACCGCAGT	CTTCTCTGATC	TTTGAATGAC	TCGAGTACAT
10561	GAGCGCACCT	TTCCGAGACTG	ATCTCTCTTG	TTCTCCATAG	GTCAGCTACG	CCCTGCTCC	GGACTCGCTA	CTGAGATACC
10641	TTTGTCTTTC	AGACTGACGC	CGCTAGAGGA	CTTCTCGGG	GAGATGAGGA	CACCGGACA	GGTGTGCTG	ACCTGTGTT
10721	ACTAGTGAGT	ACAGTGGCTG	GCTGACGTGT	CCCTGCGGAT	CTATACAAAT	AGTTCTTGTG	TCCTGACAGA	ATAGCATGT
10801	GGGAGATTG	CAAGTAACC	TAGGCTGCAC	AGGTGAGATA	AAGCTCAGAG	CAGACGGTCC	GAGCAGGAG	CAGGTAAGAC
10881	GGGGTGTTC	TCTGCACGGA	TGCTGTGAC	CGGTCTATTC	TTGGTGAAGG	GACTCTTGCT	CTCGCCGCGC	CGCCCGTCCG
10961	TTCTGCTGCT	CGTCTGATCC	ATCTCTCTTT	CTGATTGACG	CACGAGGGTC	GGGTAGCAG	CTGTAGTAG	AGTCTCTCGG
11041	GTCCGGGGG	GTGAGGGACG	CGACTGCTCA	CGGTTCTGTT	TGCTGTAGA	TATGAGCGT	GTTCGGGGAG	CTTCTCTCTC
11121	AGACTCGGTG	GGGACCCCTCC	ACGGCGATCA	CAACTACCAC	TGCATGGCGA	CTTCAAGCT	GGATTGCACT	TCGGCTCGGA
11201	CCCGGGGGT	TGTAAGTATC	CTGGAGCACA	GGGACCCCGC	GACCGTGGAC	GATATCTTTA	TGCAGCGGGT	CTGTACGAC
11281	ATCAACGACG	TGCTCGGGCC	GGTCGATGT	CTCACGAGCT	TCCCGGAATA	CAGAAGAAC	CTGAGGGTGG	CCGTGGAGGC
11361	CGCCACCGAA	CTCCACGGCG	TCTCCGCTCT	GTCCGGCCAG	CTGACCAAGT	TCTTGGAGGA	CCACGATGGA	GCGATGCTGC
11441	CCCTGGCCTG	GCCCCGGAAC	AGGTACCTTC	GCCTGACGTC	GGGCAGAAAG	CTGGTTATC	TCGACGTGGC	GGAGAGCTG
11521	CGCAAGGAAT	ACCCCATGTG	CAACGGTCCC	CTCGAGTTTC	TGCGCTGTAT	CTCGAGCCC	GCCAGTCTCC	CTACAGGCA
11601	CCAGTACCCC	GTCTCTTTGA	TGGGCTCTAC	CGGGACCATC	TTCTCTCCACG	CCAGGACCC	GGCCGCTGAG	AGTCCGACT
11681	ACGATCCGGA	GCGGAGCGCC	GATCGACTCT	TCCTGGCGCG	CGAGTCCCTG	CAGTCTTCG	GTAGGGAAGG	ACTCTGCAGG
11761	TGGGACAAACG	TCTACACCGA	GGACGGCGGC	GCTCCGCTACG	CTACTCCCGA	GGACCCCGTG	CTCAGAAACA	TCGTGTTCAC

Fig. 4

11841	GGCCCATCTC	GGCGGGAAGG	CGCTCCACCA	GCAGATCTGT	AAGATCAAGG	GACACACCTG	GTACCTGAAC	GTTGCCCCG	11920
11921	GCATGCTGAA	GGACAGGGTG	TTCGTGCGCA	CGCGGACAT	CCCCCCTTC	GTCCGCCACG	TGAACCTGGA	ACTCTTTGGA	12000
12001	CATAGGTTTC	TACCCATCGG	TAGGGTGACC	CGTTCCCCG	AGGATCCCGA	GTGTGAGATG	TTTATCATGG	TGGACGCCGG	12080
12081	GGGGGCCATC	TACGGGCACA	TGTTAGATAG	CGGGAAGGTG	AGGAGGCTAG	CTGATAACTT	TGACCAAGTTC	ATCGGGATGG	12160
12161	GCACCCGGAG	GGCTTATTTC	AATTTCAGA	TGGTTAAGGG	AGATAGTCTC	AACACCGAGT	ACGAGGAGAC	CTCGTTTGGG	12240
12241	ACCAAGAAC	ACGGGTTCTT	TCTCTTGACC	AGAGACCTGG	TGACCGTCAG	GAAGAAGATT	CAGTGAGAGG	ACACGAGGTA	12320
12321	TGTCATGGTT	TTTAATCAAT	AAATAAGAG	GTTTATTCA	TCGGACAGTC	GTTGTAGCCT	GTAAAGACT	CGCCCCGGAG	12400
12401	GGGGTTCCCC	CGATGTGAGG	GGCATGCAGT	AGTATGGTGT	CCTGAGTGTC	TCGGATGCCG	TCTTGAACCTC	GCACTCTACC	12480
12481	GCCGTGGGGG	TTAATAAGT	TTTTGCTGG	CCGGTAGGGG	GGGAGGCCGA	GGATAATAAA	GTTGCTACGT	ACTGGTTGAA	12560
12561	GTCTAACAAAT	CTCTCGGGG	GATCCACGGT	CCACCGTCTC					12600

HinDIIINruITATA.....ATGRestriction sites and initiation codons for MCMV ie2Fig. 4, cont.

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The HindIII-L restriction enzyme digestion fragment of MCMV is cloned into the plasmid vector pGEM 11Zf. This host plasmid is then used to clone other genes or DNA sequences into the HpaI restriction enzyme site as part of the engineering process.

Fig. 5

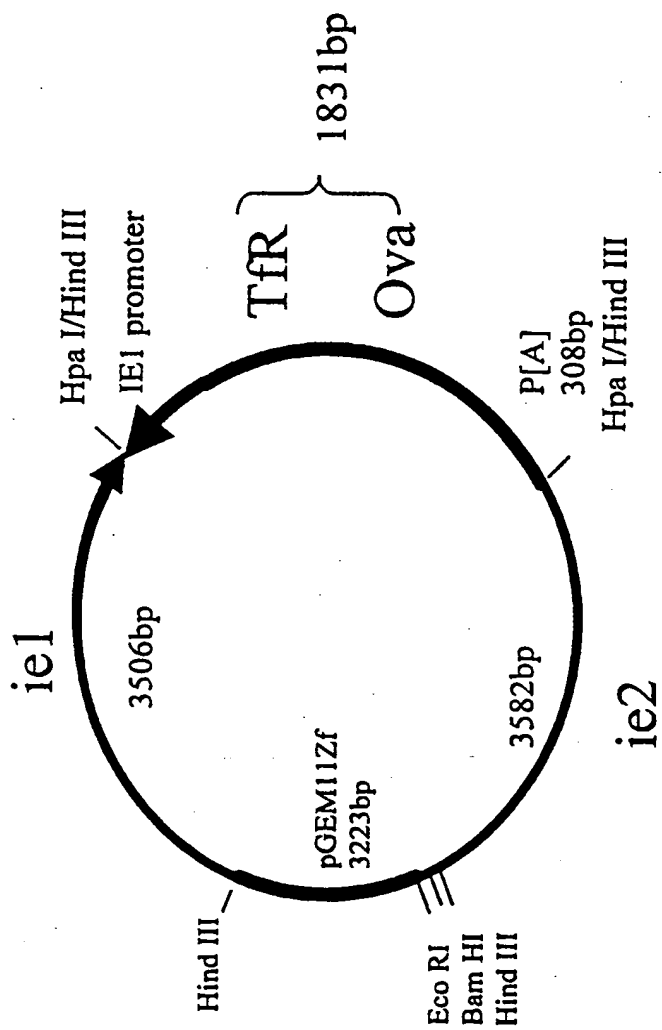


Fig. 6a.

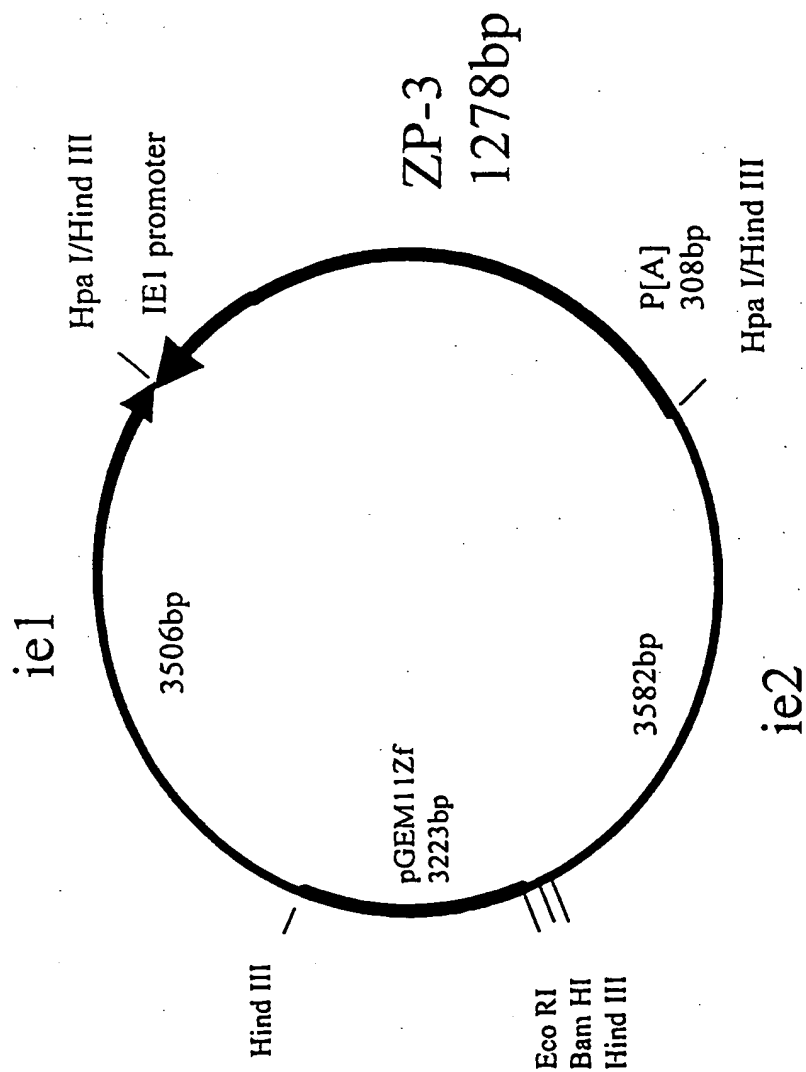
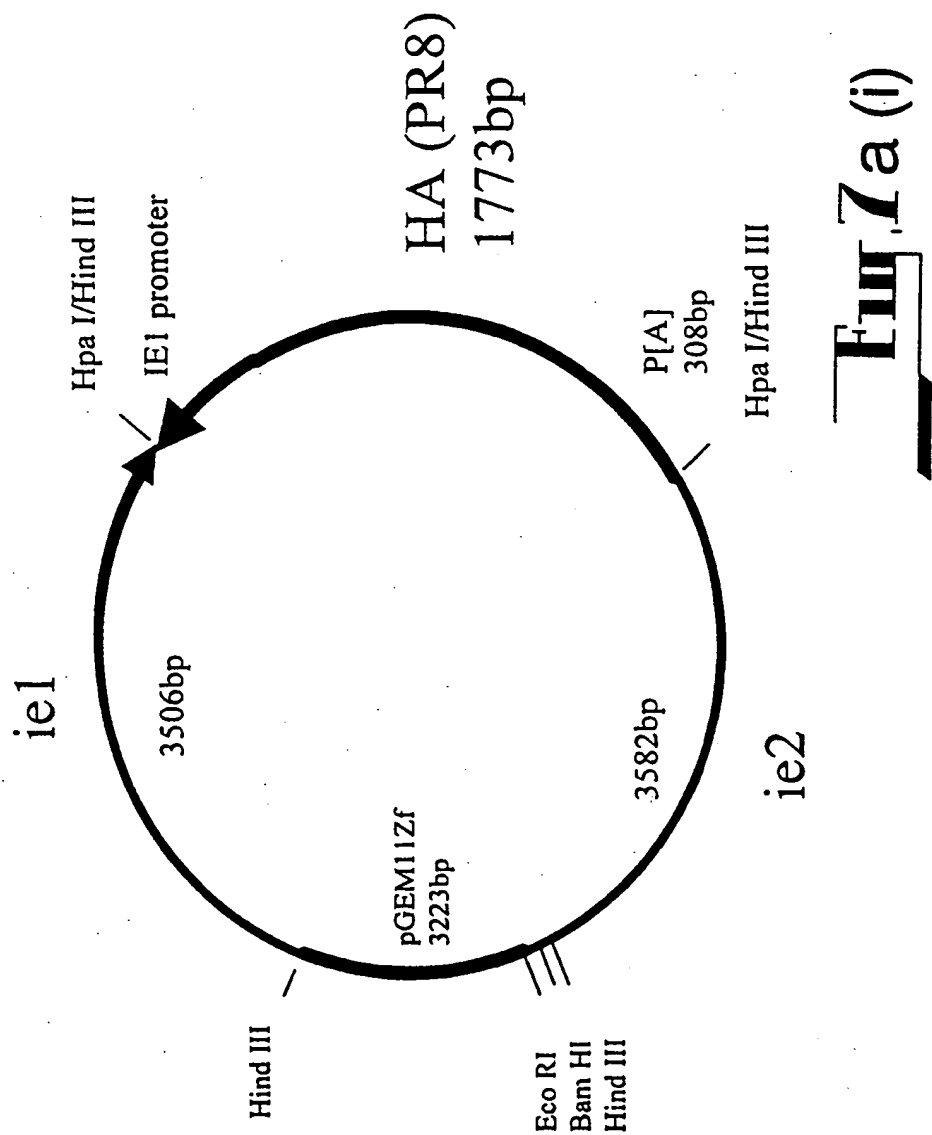


Fig. 6b

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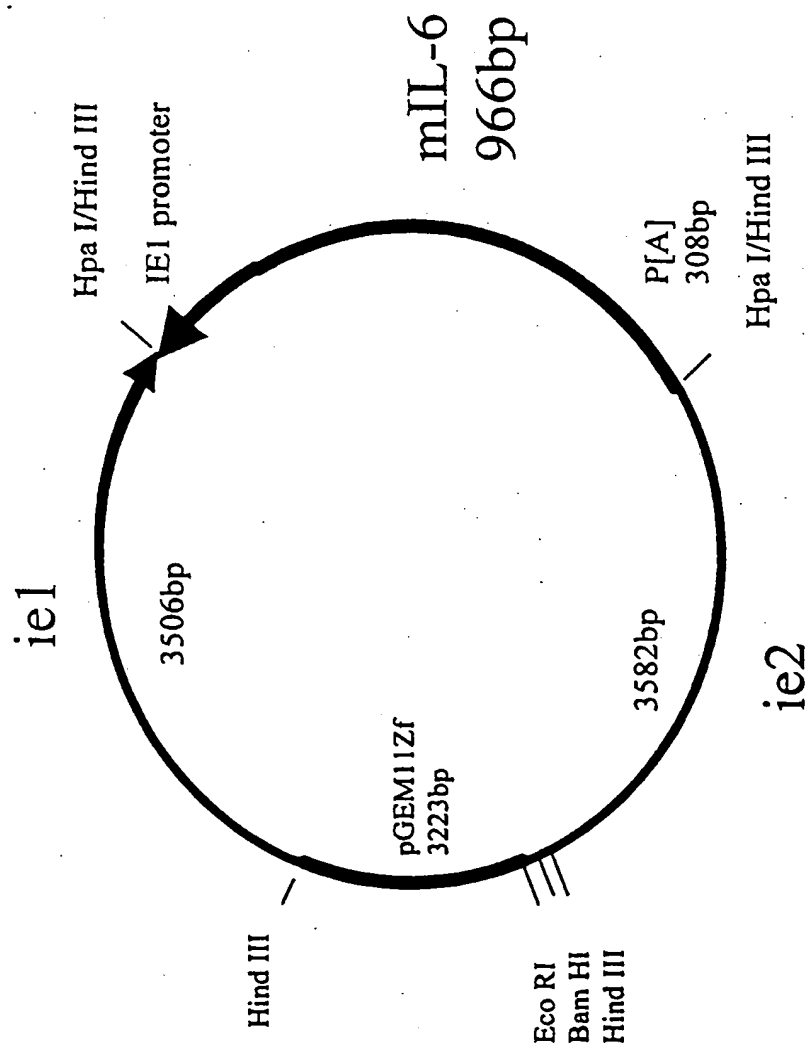


Fig. 7a(ii)

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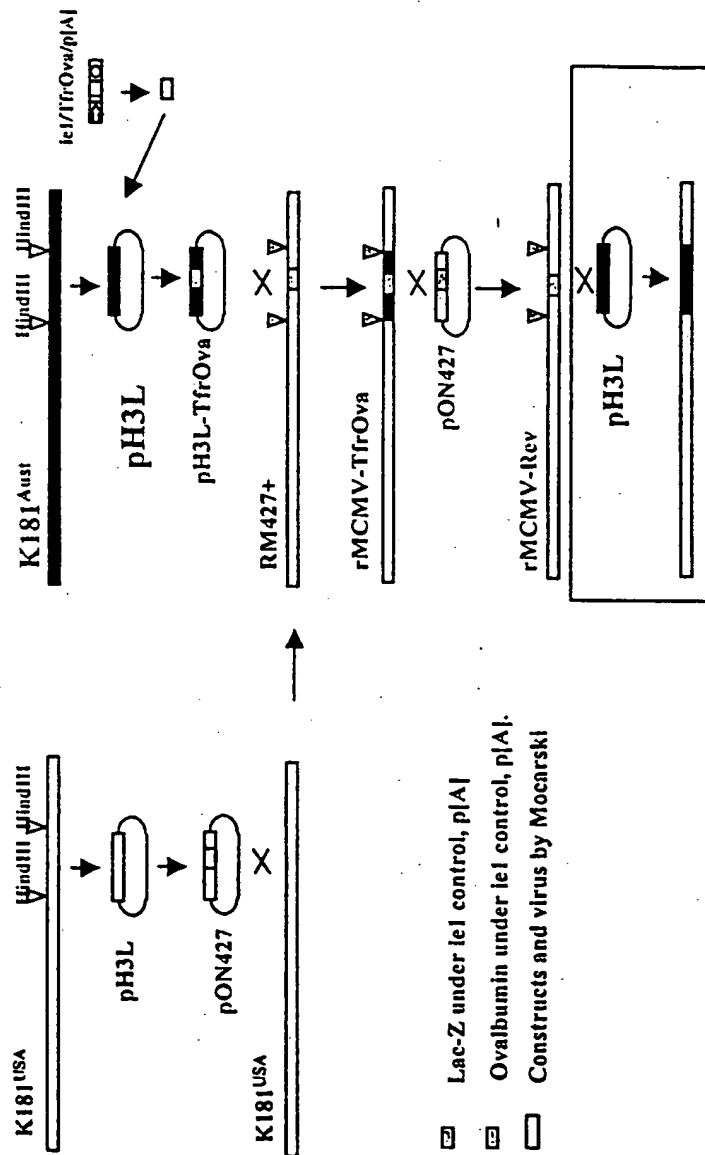
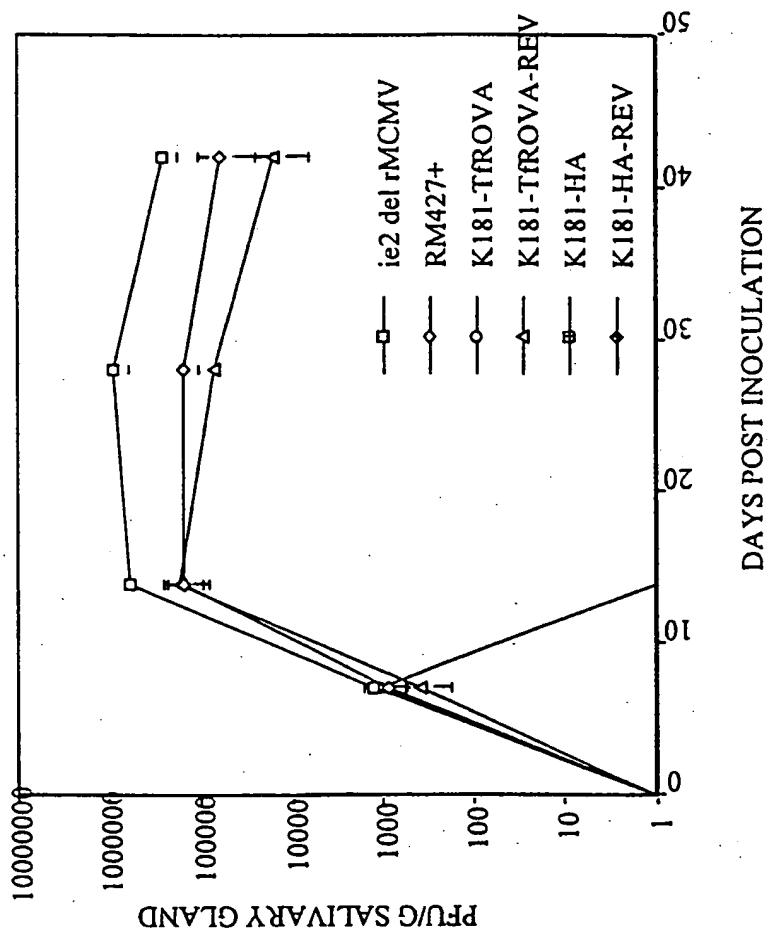


Fig. 7b,

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**Fig. 8**

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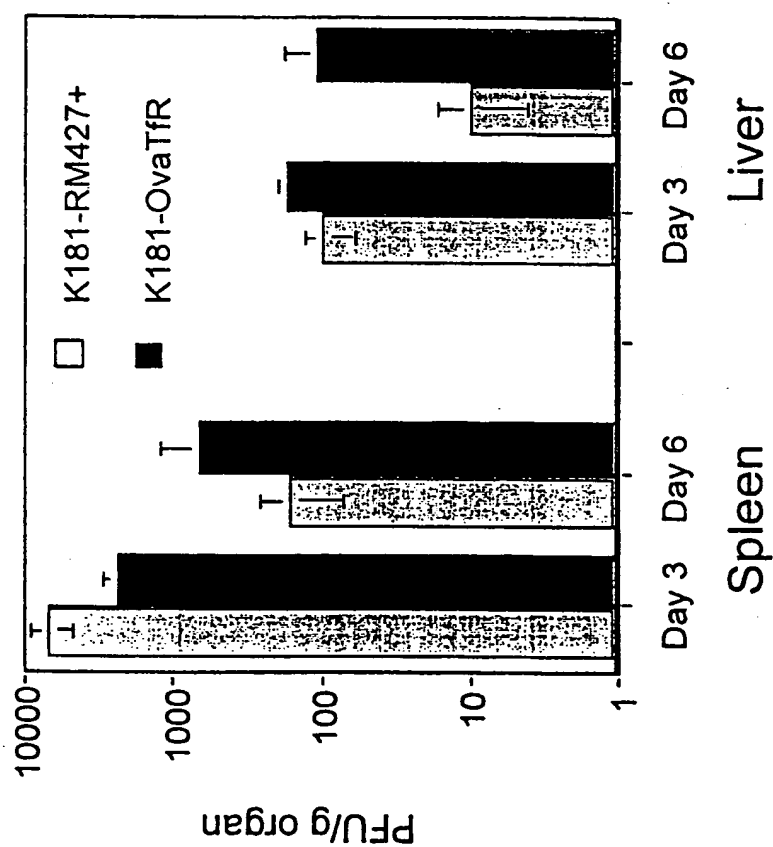


Fig. 9

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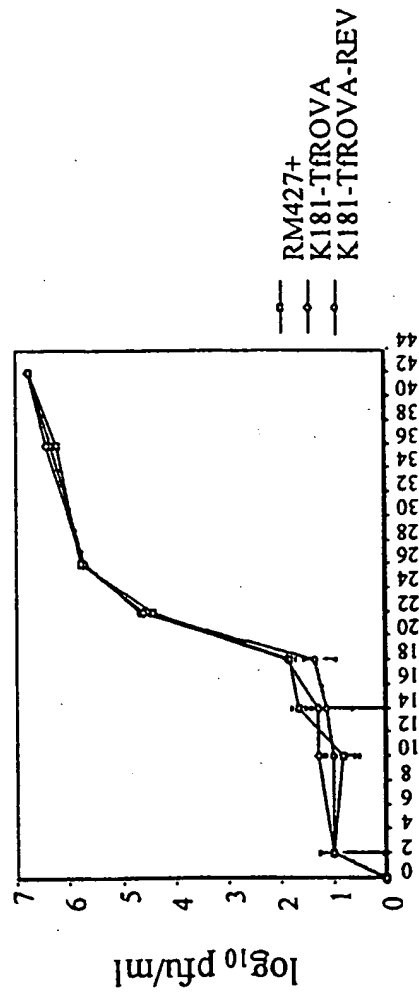


Fig. 10a

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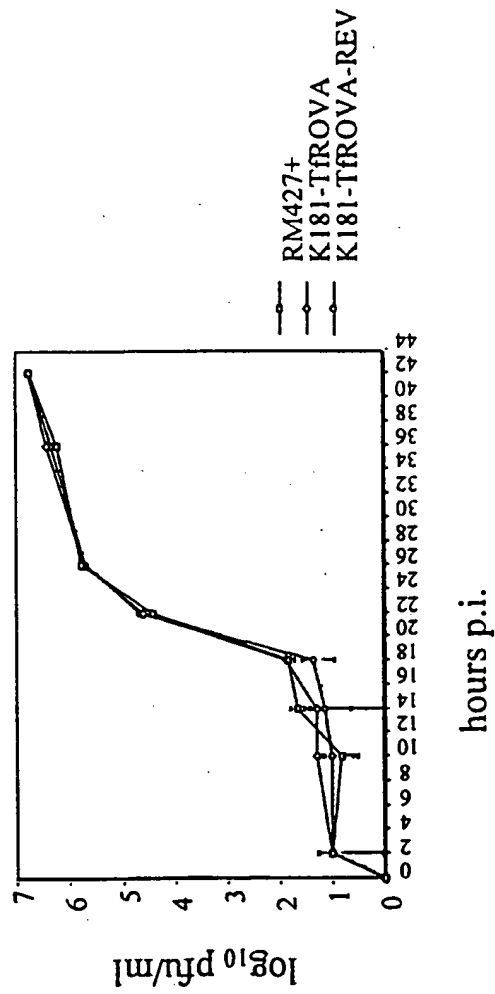


Fig. 10b

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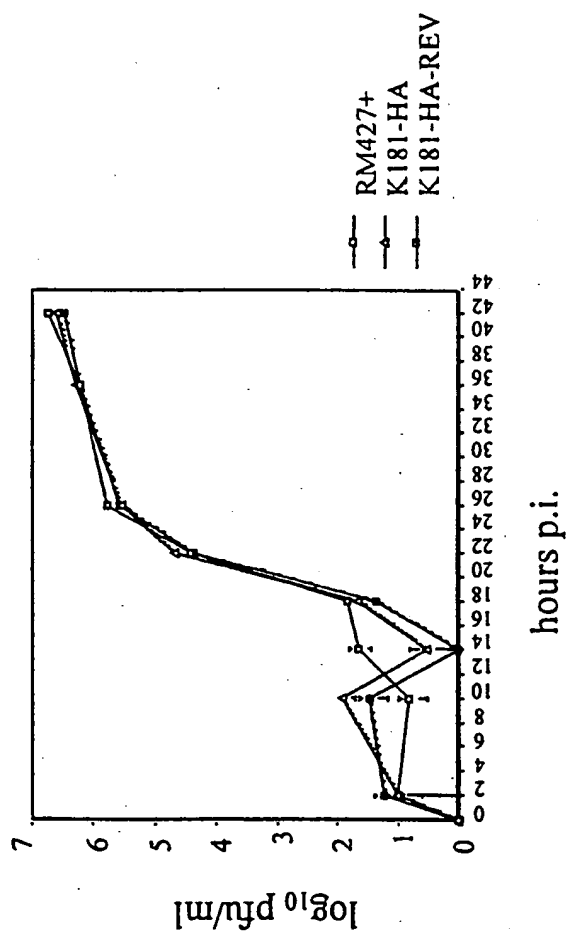
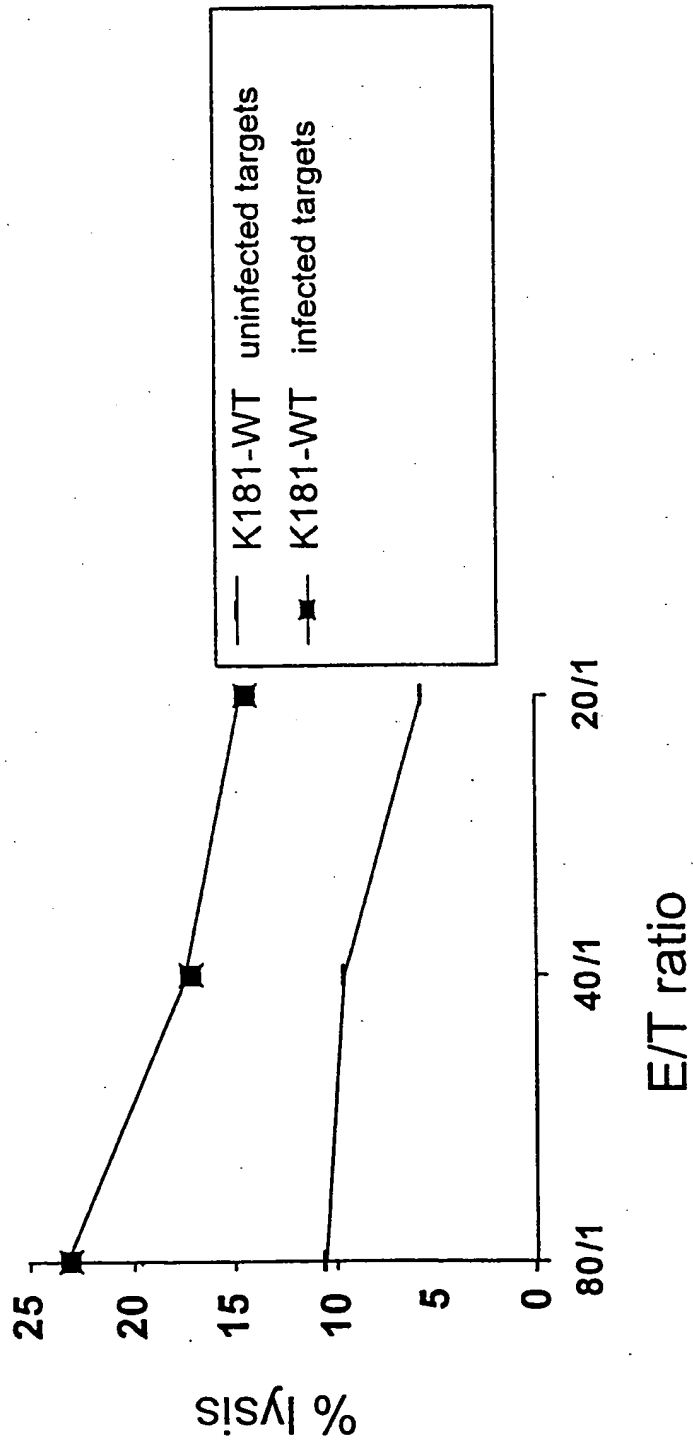


Fig. 10c.

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**Fig. 11**

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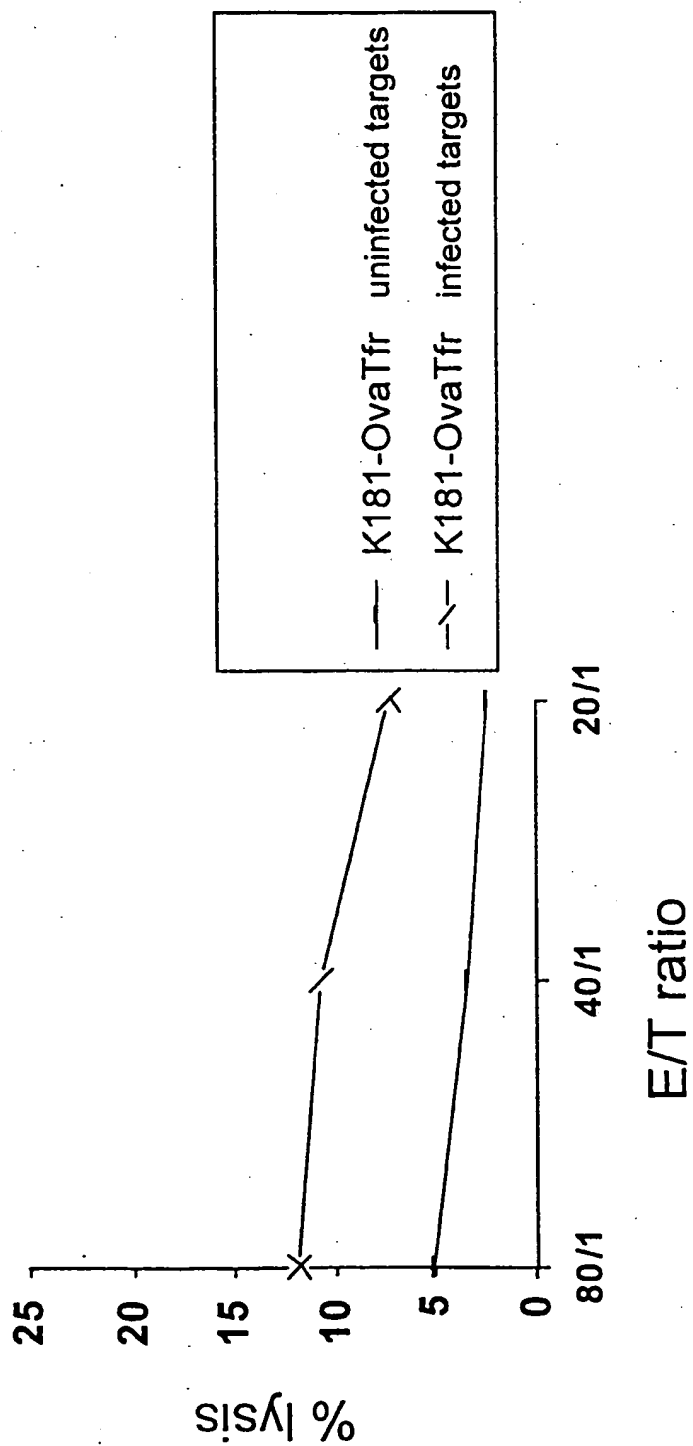


Fig. 12

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Summary Results of i.p inoculations with 2×10^4 TCV.

MCMV-ZP3	BALB/c +/- spleen and liver, d3 nothing at other time points (7, 21, 35, 100)
	C57BL/6 nothing at any time point (3, 7, 21, 35, 100)
	CBA +/- spleen d3 nothing at other time points (7, 21, 35, 100)
	alpha R -/- +/- spleen and liver, d3 +/- Sal. Gland d6 nothing at other time points (d10)
MCMV-LacZ+	BALB/c +/- spleen and liver, d3 + SG d7 ++++ SG d21, 28, 49 nothing at other time points (d100)
	C57BL/6 nothing at any time point (3, 7, 21, 35, 100)
	Bg/Bg nothing at any time point (3, 7)
	alpha R -/- +/- spleen and liver, d3 +/- spleen and liver, d6
	Gamma R -/- +/- spleen and liver, d6
Alpha/Gamma R -/- Lethal d8	
MCMV-HA	BALB/c nothing at any time point (4, 7, 21, 35, 100)
	C57BL/6 nothing at any time point (4, 7, 21, 35, 100)
Alpha/Gamma R -/- Lethal d8	
MCMV-OVA	alpha R -/- nothing at time points d3, 6
	BALB/c +/- spleen and liver, d3 nothing at other time points (7, 21, 35, 100)
	C57BL/6 nothing at any time point (3, 7, 21, 35, 100)
	alpha R -/- + spleen and liver, d3 ++ spleen and liver, d6
	Gamma R -/- +/- spleen and liver, d6 +/- spleen and liver, d6
	Nu/Nu nothing at day 10
Bg/Bg +/- spleen and liver, d3 nothing at other time points (7, 17)	
Alpha/Gamma R -/- Lethal d8	

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K181-WT

days p.i.	<u>Anti-MCMV Ab titres</u>	
	IgG1	IgG2a
7	20	<10
14	<10	20
28	<10	160
41	<10	320

RM427+

days p.i.	<u>Anti-MCMV Ab titres</u>		<u>Anti-Bgal Ab titres</u>	
	IgG1	IgG2a	IgG1	IgG2a
7	<10	<10	<10	<10
14	20	10	<10	40
28	40	10	<10	40
49	20	10	20	80

K181-TiROVA

days p.i.	<u>Anti-MCMV Ab titres</u>		<u>Anti-ovalbumin Ab titres</u>	
	IgG1	IgG2a	IgG1	IgG2a
7	<10	<10	<10	20
14	<10	<10	20	80
28	<10	10	40	160
49	10	10	40	160
105	10	10	40	160

Fig. 14a.

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	Day p.i.	IgM	IgG1	IgG2a
K181-HA	29	80	<10	160
	39	80	<10	160
K181-HA 1°				
D33, K181-HA 2°	29	40	<10	160
	39	320	<10	>1280
	47	160-320	<10	>1280

Anti-MCMV Antibody Response

		IgM	IgG1	IgG2a
Day 7	Ovalbumin Protein	0	20	0
	K181-OVATfR	0	40	0
	UV-inactivated	0	20	0
Day 17	Ovalbumin Protein	0	20	20
	K181-OVATfR	0	20	160
	UV-inactivated	0	40	20

Anti-Ovalbumin Antibody response

		IgM	IgG1	IgG2a
Day 7	Ovalbumin Protein	0	0	0
	K181-OVATfR	0	20	0
	UV-inactivated	0	0	0
Day 17	Ovalbumin Protein	0	20	0
	K181-OVATfR	0	20	80
	Uv-inactivated	0	20	0

Fig. 14b

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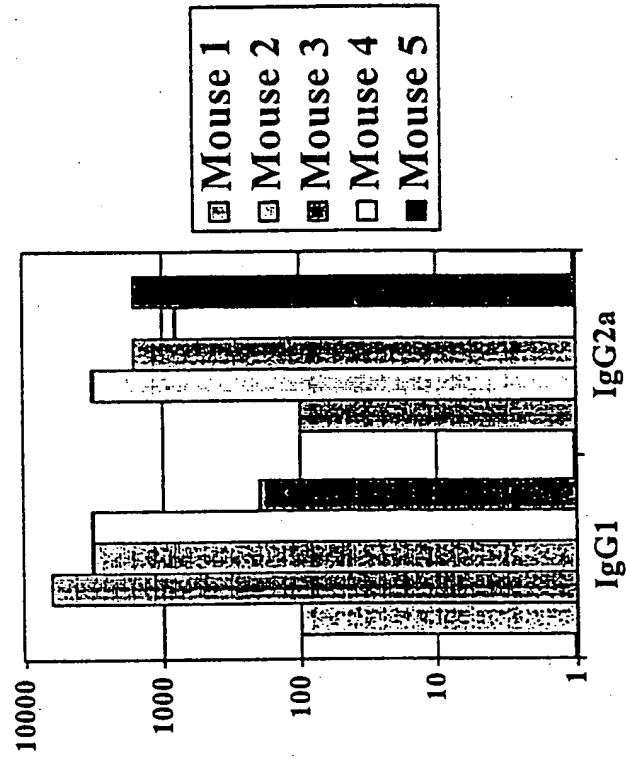


Fig. 15

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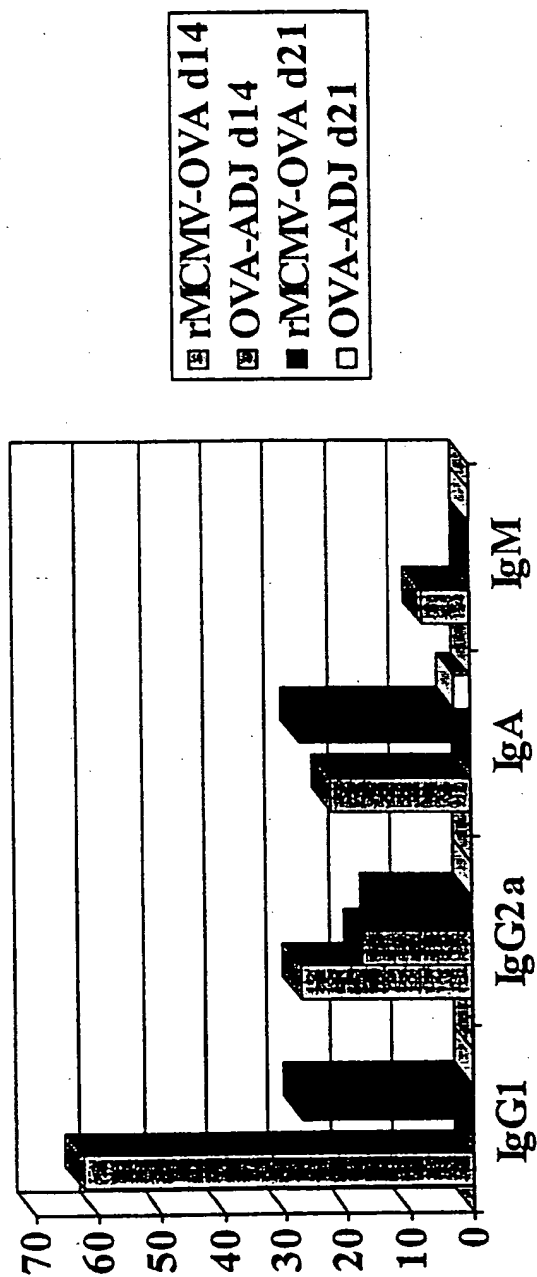


Fig. 16

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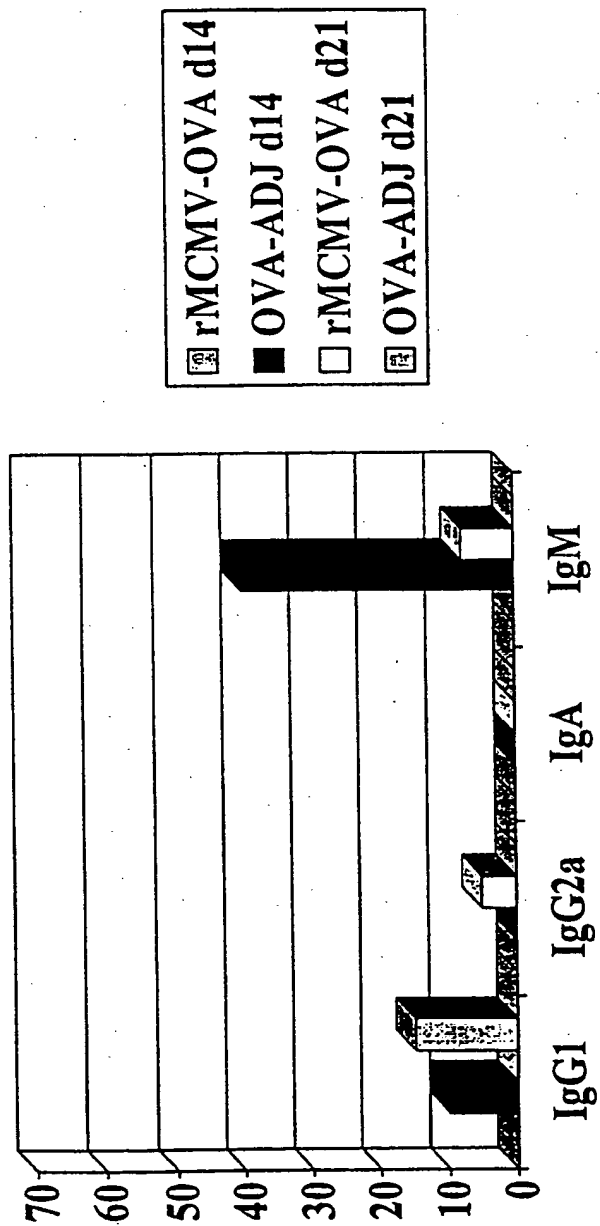


Fig. 17

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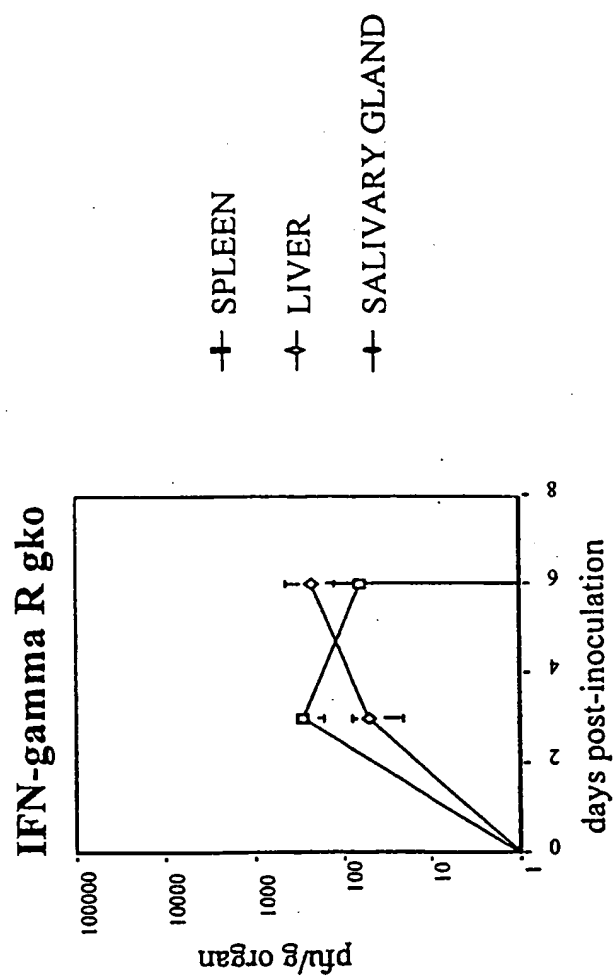


Fig. 18a

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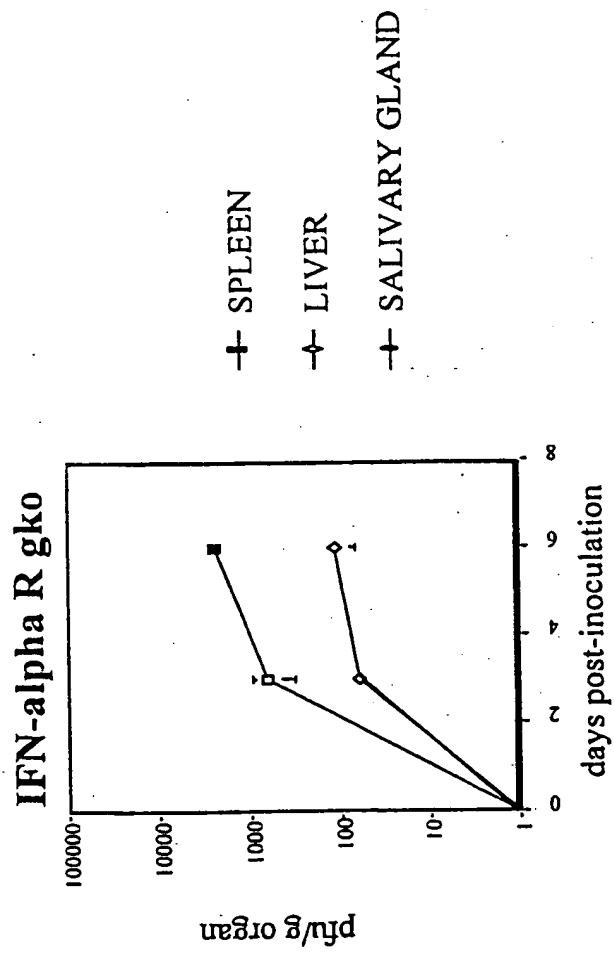


Fig. 18b

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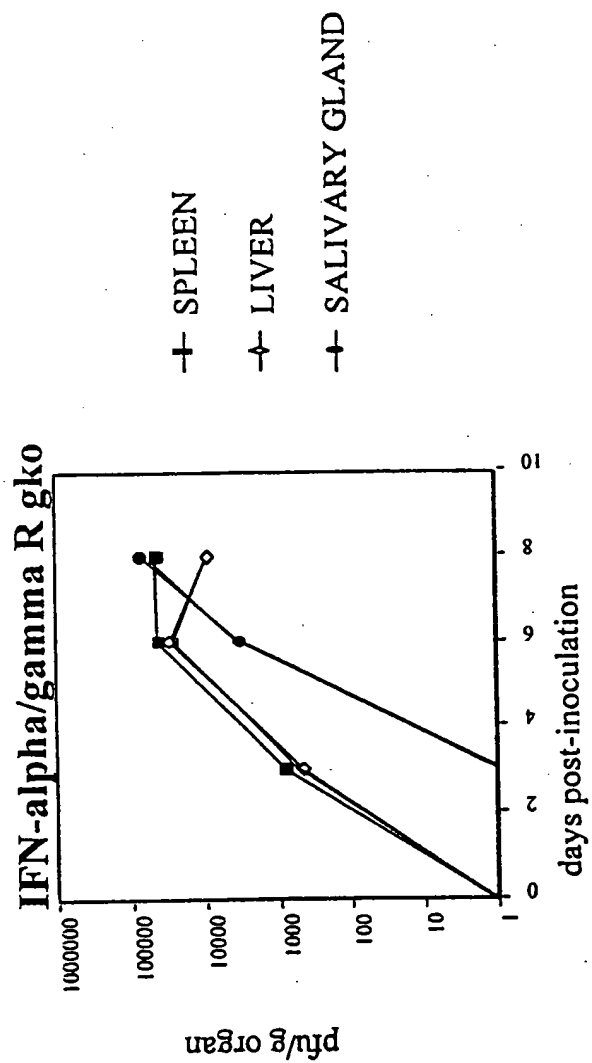
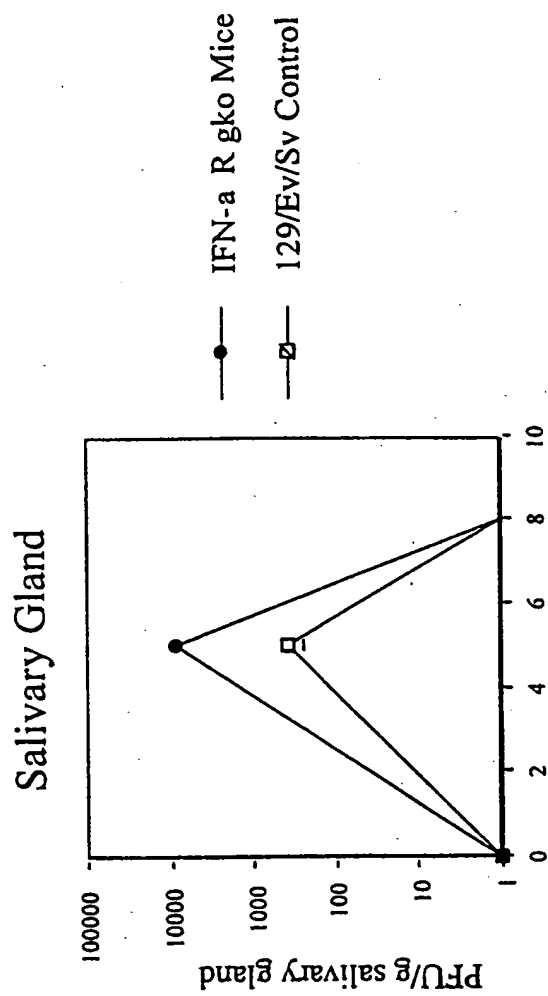


Fig. 18c

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**Fig. 19a.**

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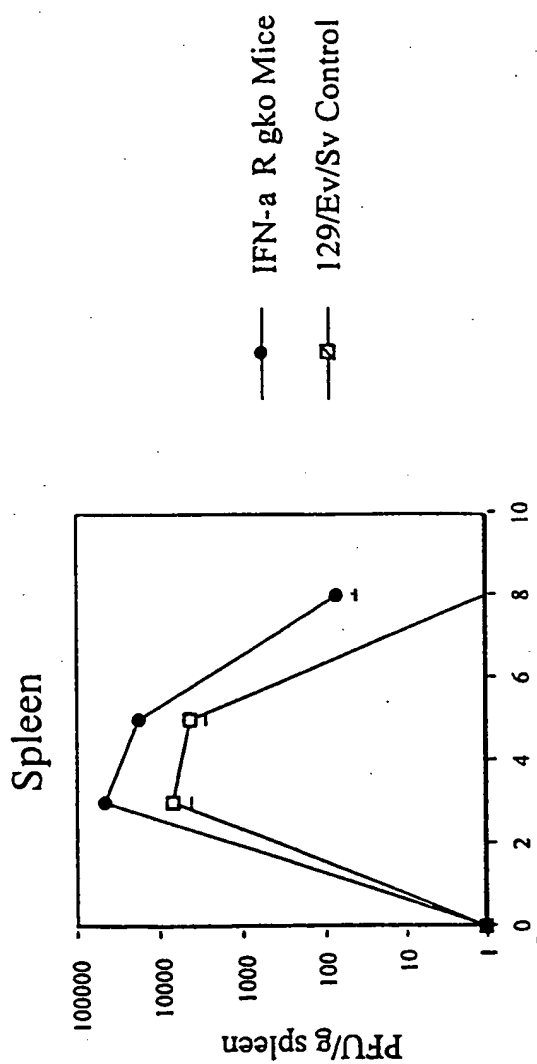


Fig. 19b

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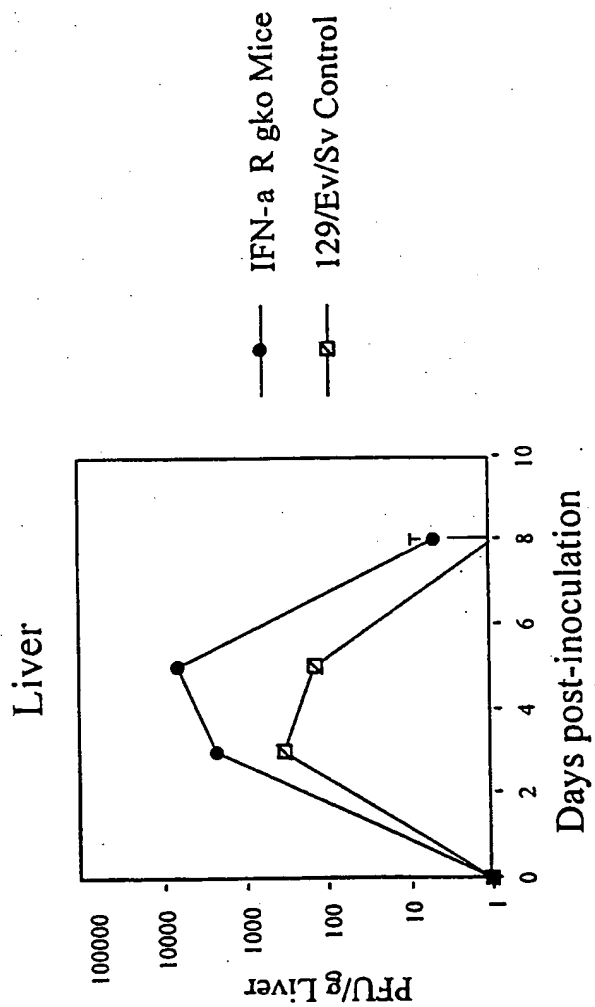


Fig. 19c

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rK181-IL6Biologically active *in vitro*BALB/c and C57BL/6 mice inoculated with 2×10^4 pfu rK181-IL6PLAQUE ASSAY

<u>BALB/c</u>	Day3	Day7	Day21	Day35
Spleen	-	-	-	ND
Liver	-	-	-	ND
Sal.Gland	ND	-	$1.9 \times 10^3^*$	$7.5 \times 10^3^*$

<u>C57BL/6</u>	Day3	Day7	Day21	Day35
Spleen	-	-	-	ND
Liver	-	-	-	ND
Sal.Gland	ND	-	-	-

ND: Not Done

* pfu/gram organ

Fig. 20.

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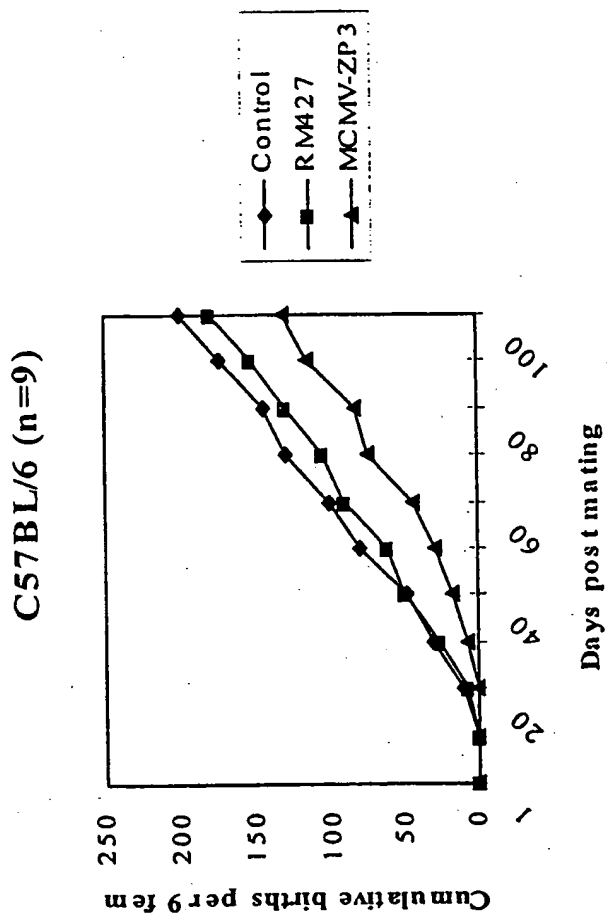


Fig 21

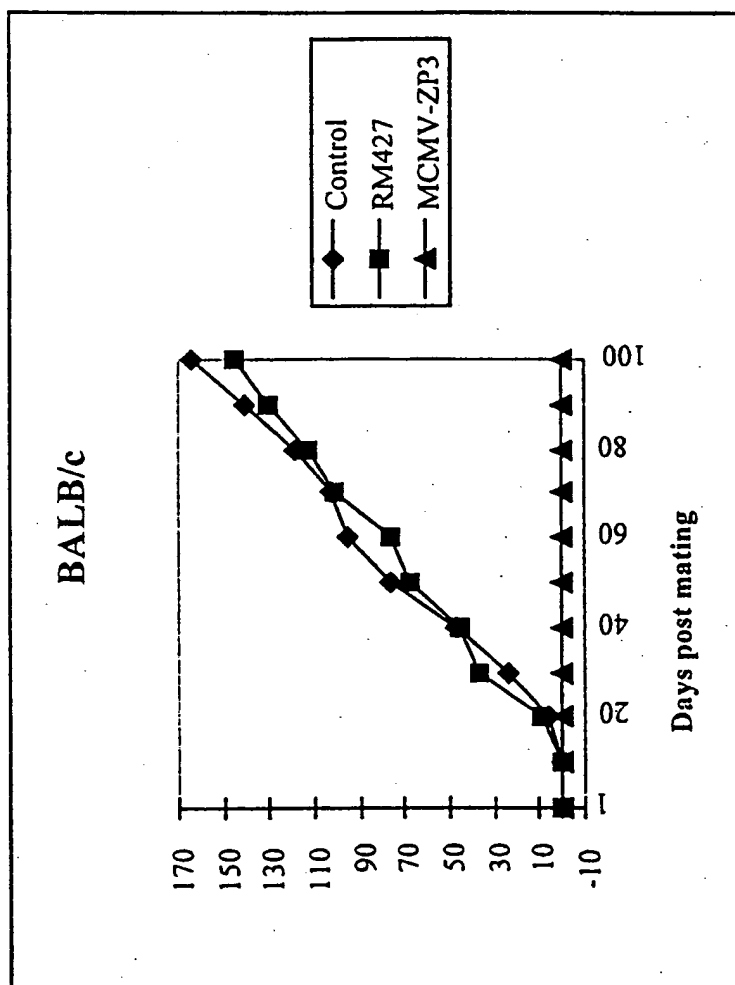


Fig. 22

ARC/s		#litters/ #females	#live births	Average litter size
Control		10/10	106*	10.6
MCMV-LacZ		10/10	130	13
MCMV-ZP3		3/9	16	5

Fig. 23.

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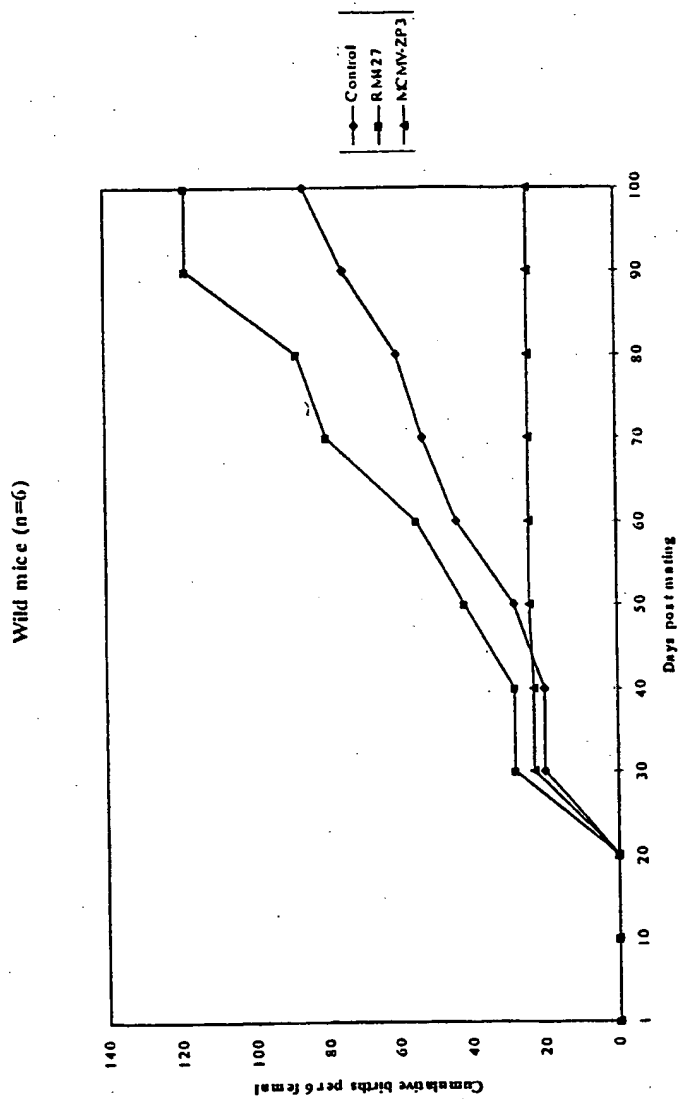


Fig. 24

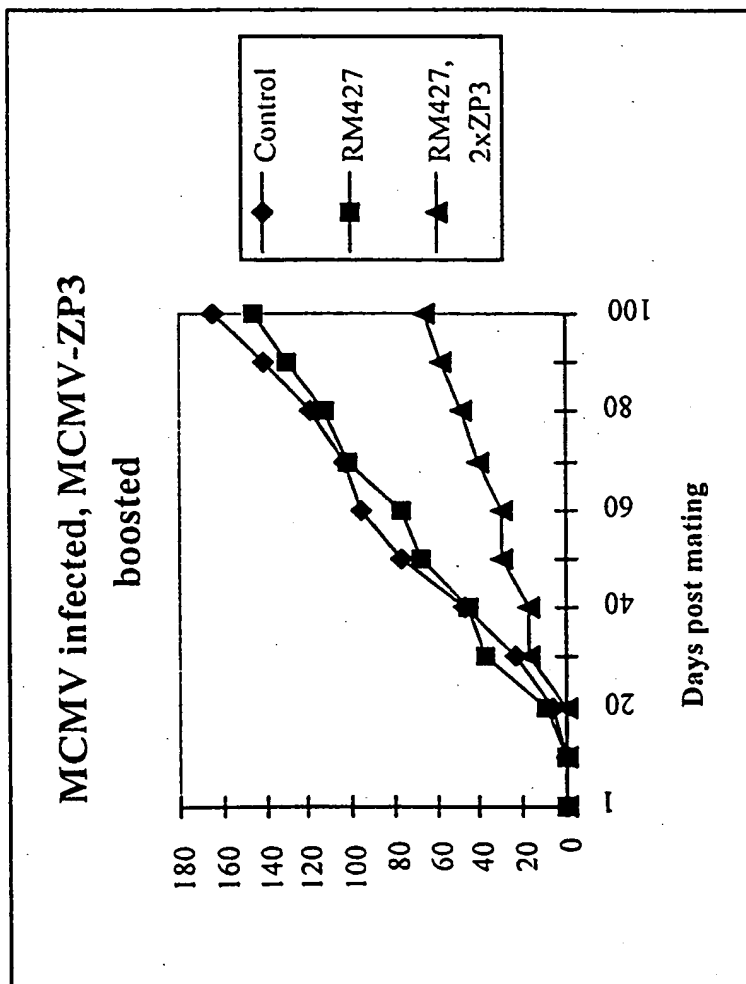


Fig. 2.5

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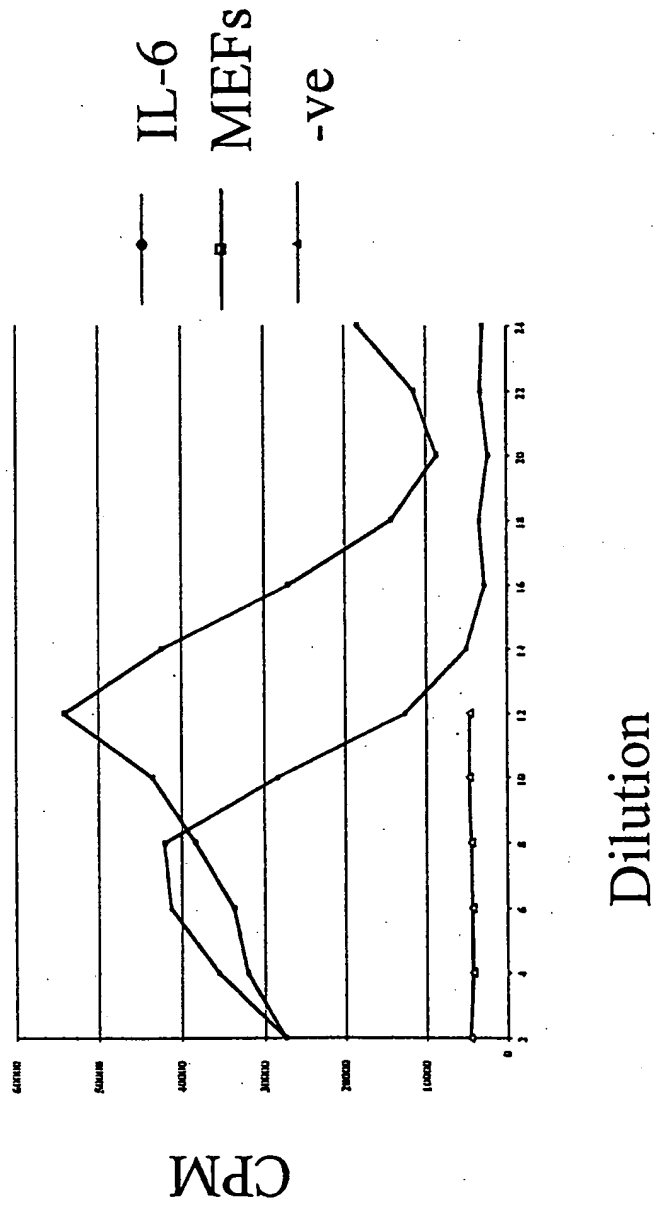


Fig. 26.

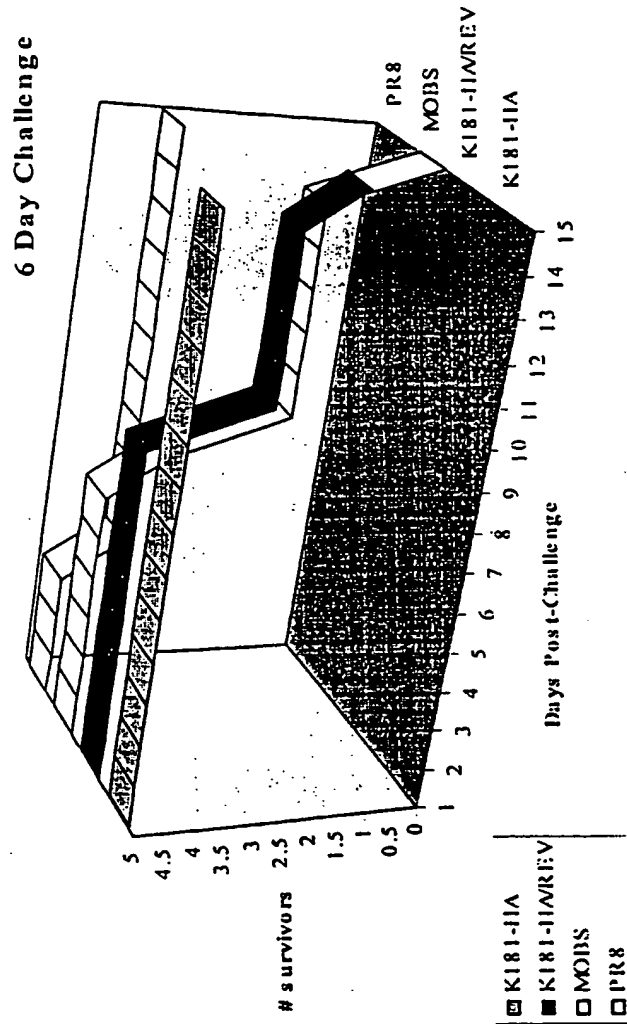


Fig. 27

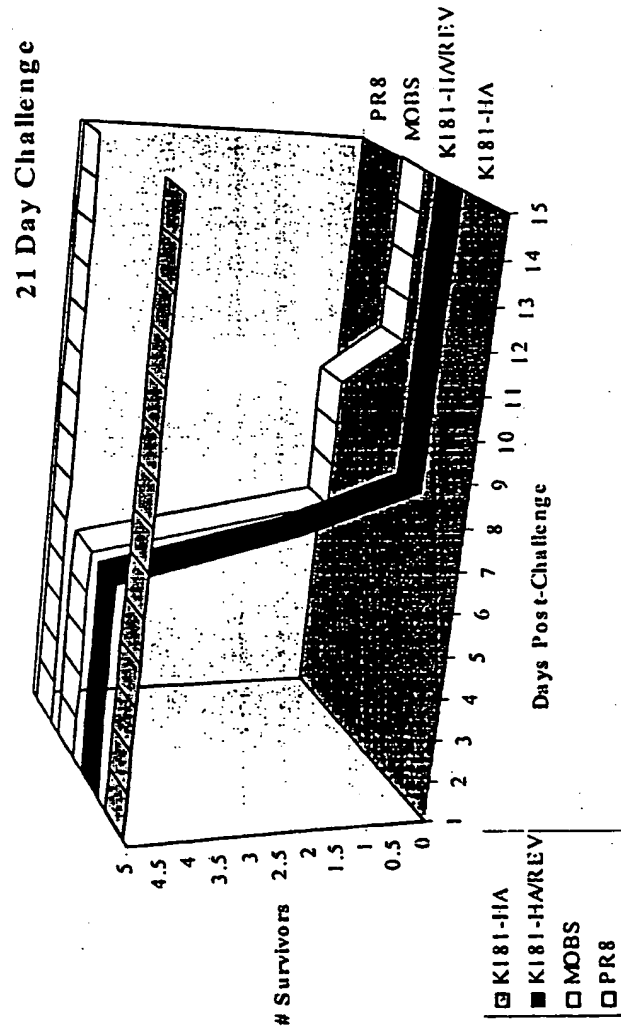


Fig. 28.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 99/00395

A. CLASSIFICATION OF SUBJECT MATTER		
Int Cl ⁶ : C12N 15/86 7/01 A61K 35/76		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched SEE ELECTRONIC DATABASES		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPAT: (Cytomegalovirus or CMV or HCMV) (5N) Vector CAS and Medline: Vector and Recombin? and genom? and cytomegalovirus		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 88/10311 (SYNTRO CORPORATION) (29 DECEMBER 1988) Claim 12	1 - 29
X	"Inducible expression of a foreign gene inserted into the human cytomegalovirus genome" M. Takekoshi et al, <i>J Gen Virol</i> , 74 (1993) pages 1649 - 1652 Whole Document	1 - 29
X	"Site-specific stable insertion into the unman cytomegalovirus genome of a foreign gene under control of the SV40 promoter" M. Takekoshi et al, <i>Gene</i> , 101 (1991) pages 209 - 213 Whole Document	1 - 29
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
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Date of the actual completion of the international search 10 June 1999		Date of mailing of the international search report 16 JUN 1999
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929		Authorized officer ALISTAIR BESTOW Telephone No.: (02) 6283 2450

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 99/00395

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	"Use of Glycoprotein gB Promoter for Expression of Genes Inserted into the Human Cytomegalovirus Genome" M. Takekoshi et al, <i>Tokai J Exp Clin Med</i> , 23(1) (1998) pages 37 - 42 Whole Document	1 - 29
X	"Cloning and Structural Analysis of Four Genes Encoding Interferon- ω in Rabbit" M. Charlier et al, <i>J Interferon Res</i> , 13 (1993), pages 313 -322 Whole Document	1 - 29

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU 99/00395

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Patent Document Cited in Search Report				Patent Family Member			
WO	88/10311	AU	20723/88	CA	1 327 171	EP	319 570
		US	5 273 876	US	5 561 063	US	5 830 745
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